

WO 92/01471(1)
C07K15/00F12B14F-[M07K201:00]-
[M07K201:02]-[M07K203:00]-
[M07K207:00]-

WORLD INTELLECTUAL
PROPERTY ORGANIZATION
BUREAU



THE PATENT COOPERATION TREATY (PCT)

14/135
- 1- *-C07K15/00F12B14F

A61K 39/00, C07H 15/12
C12N 1/00, 1/16, 5/00
C12N 7/00, C07K 3/00, 13/00
C12P 21/06, C12Q 1/70

A1

International Publication Number:

WO 92/01471

(43) International Publication Date:

6 February 1992 (06.02.92)

(21) International Application Number: PCT/US91/05194

(22) International Filing Date: 23 July 1991 (23.07.91)

(30) Priority data:
557,267 24 July 1990 (24.07.90) US

(71) Applicant: THE UAB RESEARCH FOUNDATION [US/
US]; University Station, Birmingham, AL 35294 (US).

(72) Inventors: WERTZ, Gail, W. ; 2845 Argyle Road, Bir-
mingham, AL 35213 (US). LERCH, Robert ; 2444 Cha-
let Gardens Court, Apt. 1, Madison, WI 53711 (US).

(74) Agent: MISROCK, S., Leslie; Pennie & Edmonds, 1155
Avenue of the Americas, New York, NY 10036 (US).

(81) Designated States: AT (European patent), AU, BE (Euro-
pean patent), CA, CH (European patent), DE (Euro-
pean patent), DK (European patent), ES (European pa-
tent), FI, FR (European patent), GB (European patent),
GR (European patent), HU, IT (European patent), JP,
LU (European patent), NL (European patent), NO, SE
+ (European patent), SU.

Published

With international search report.

*Before the expiration of the time limit for amending the
claims and to be republished in the event of the receipt of
amendments.*

(54) Title: METHODS OF USE OF BOVINE RESPIRATORY SYNCYTIAL VIRUS RECOMBINANT DNA, PROTEINS
VACCINES, ANTIBODIES, AND TRANSFORMED CELLS

(57) Abstract

The present invention relates to recombinant DNA molecules which encode bovine respiratory syncytial (BRS) virus proteins, to BRS virus proteins, and peptides and to recombinant BRS virus vaccines produced therefrom. It is based, in part, on the cloning of substantially full length cDNAs which encode the entire BRS virus G, F, and N proteins. According to particular embodiments of the invention, DNA encoding a BRS virus protein or peptide may be used to diagnose BRS virus infection, or, alternatively, may be inserted into an expression vector, including, but not limited to, vaccinia virus as well as bacterial, yeast, insect, or other vertebrate vectors. These expression vectors may be utilized to produce the BRS virus protein or peptide in quantity; the resulting substantially pure viral peptide or protein may be incorporated into subunit vaccine formulations or may be used to generate monoclonal or polyclonal antibodies which may be utilized in diagnosis of BRS virus infection or passive immunization. In additional embodiments, BRS virus protein sequence provided by the invention may be used to produce synthetic peptides or proteins which may be utilized in subunit vaccines, or polyclonal or monoclonal antibody production. Alternatively, a nonpathogenic expression vector containing the genes, parts of the genes, any combination of the genes, or parts thereof may itself be utilized as a recombinant virus vaccine.

+ DESIGNATIONS OF "SU"

It is not yet known for which States of the former Soviet Union any designation of the Soviet Union has effect.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	SD	Sudan
CG	Congo	KP	Democratic People's Republic of Korea	SE	Sweden
CH	Switzerland	KR	Republic of Korea	SN	Senegal
CI	Côte d'Ivoire	LI	Liechtenstein	SU+	Soviet Union
CM	Cameroon	LK	Sri Lanka	TD	Chad
CS	Czechoslovakia	LU	Luxembourg	TC	Togo
DE	Germany	MC	Monaco	US	United States of America
DK	Denmark				

METHODS OF USE OF BOVINE RESPIRATORY SYNCYTIAL VIRUS RECOMBINANT DNA,
PROTEINS VACCINES, ANTIBODIES, AND TRANSFORMED CELLS.

1. INTRODUCTION

The present invention relates to recombinant DNA molecules which encode bovine respiratory syncytial (BRS) virus proteins as well as corresponding BRS virus proteins and peptides derived therefrom. It is based, in part, on the cloning of full length cDNAs encoding a number of bovine respiratory syncytial virus proteins, including, F, G, and N. DNAs encoding the G and F proteins have been inserted into vaccinia virus vectors, and these vectors have been used to express the G and F proteins in culture and G protein encoding vectors have been used to induce an anti-bovine respiratory syncytial virus immune response. The molecules of the invention may be used to produce safe and effective bovine respiratory syncytial virus vaccines.

2. BACKGROUND OF THE INVENTION

2.1. BOVINE RESPIRATORY SYNCYTIAL VIRUS

Bovine respiratory syncytial (BRS) virus strain 391-2 was isolated from an outbreak of respiratory syncytial virus in cattle in North Carolina during the winter of 1984 to 1985. The outbreak involved five dairy herds, a beef calf and cow operation, and a dairy and steer feeder operation (Fetrow et al., North Carolina State University Agric. Extension Service Vet. Newsl.).

Respiratory syncytial virus, an enveloped, single-stranded, negative-sense RNA virus (Huang and Wertz, 1982, J. Virol. 43:150-157; Kingsbury et al., 1978, Intervirology 10:137-153), was originally isolated from a chimpanzee (Morris, et al., 1956, Proc. Soc. Exp. Biol. Med. 92:544-549). Subsequently, respiratory syncytial virus has been isolated from humans, cattle, sheep and goats (Chanock et al. 1957, Am. J. Hyg. 66:281-290; Evermann et al., 1985, AM. J. Vet. Res. 46:947-951; Lehmkuhl et al., 1980, Arch. Virol. 65:269-276; Lewis, F.A., et al., 1961, Med. J. Aust.

48:932-33; Paccaud and Jacquier, 1970, Arch. Gesamte Virusforsch 30:327-342). Human respiratory syncytial (HRS) virus is a major cause of severe lower respiratory tract infections in children during their first year of life, and epidemics occur annually (Stott and Taylor, 1985, Arch. Virol. 84:1-52). Similarly, BRS virus causes bronchiolitis and pneumonia in cattle, and there are annual winter epidemics of economic significance to the beef industry (Bohlender et al., 1982, Mod. Vet. Pract. 63:613-618; Stott and Taylor, 1985, Arch. Virol. 84:1-52; Stott et al., 1980, J. Hyg. 85:257-270). The highest incidence of severe BRS virus-caused disease is usually in cattle between 2 and 4.5 months old. The outbreak of BRS virus strain 391-2 was atypical in that the majority of adult cows were affected, resulting in a 50% drop in milk production for one dairy herd and causing the death of some animals, while the young of the herds were only mildly affected (Fetrow et al., 1985, North Carolina State University Agric. Extension Service Vet. Newsl.).

BRS virus was first isolated in 1970 (Paccaud and Jacquier, 1970, Arch. Gesamte Virusforsch. 30:327-342), and research has focused on the clinical (van Nieuwstadt, A.P. et al., 1982, Proc. 12th World Congr. Dis. Cattle 1:124-130; Verhoeff et al., 1984, Vet. Rec. 114:288-293) and pathological effects of the viral infection on the host (Baker et al., 1986, J. Am. Vet. Med. Assoc. 189:66-70; Castleman et al., 1985, Am. J. Vet. Res. 46:554-560; Castleman et al. 1985, Am. J. Vet. Res. 46:547-553) and on serological studies (Baker et al., 1985, Am. J. Vet. Res. 46:891-892; Kimman et al., 1987, J. Clin. Microbiol. 25:1097-1106; Stott et al., 1980, J. Hyg. 85:257-270). The virus has not been described in molecular detail. Only one study has compared the proteins found in BRS virus-infected cells with the proteins found in HRS virus-infected cells (Cash et al., 1977, Virology 82:369-379). In contrast, a

detailed molecular analysis of HRS virus has been undertaken. cDNA clones to the HRS virus mRNAs have been prepared and used to identify 10 virus-specific mRNAs which code for 10 unique polypeptides, and the complete nucleotide sequences for 9 of the 10 genes are available
5 (Collins, P.L., et al., 1986, in "Concepts in Viral Pathogenesis II," Springer-Verlag., New York; Stott and Taylor, 1985, Arch. Virol. 84:1-52).

Two lines of evidence suggest that HRS virus and BRS virus belong in distinct respiratory syncytial virus
10 subgroups. First, BRS virus and HRS virus differ in their abilities to infect tissue culture cells of different species (Paccaud and Jacquier, 1970, Arch. Gesamte Virusforsch. 30:327-342). With one exception, studies have shown that BRS virus exhibits a narrower host range than
15 HRS virus. Matumoto et al. (1974, Arch. Gesamte Virusforsch. 44:280-290) reported that the NMK7 strain of BRS virus has a larger host range than the Long strain of HRS virus. Others have been unable to repeat this with
20 other BRS strains (Paccaud and Jacquier, 1970, Arch. Gesamte Virusforsch. 30:327-342; Pringle and Crass, 1978, Nature (London) 276:501-502). The second line of evidence indicating that BRS virus differs from HRS virus comes from the demonstration of antigenic differences in the major
25 glycoprotein, G, of BRS virus and HRS virus (Orvell et al., 1987, J. Gen. Virol. 68:3125-3135). Studies using monoclonal antibodies have grouped HRS virus strains into two subgroups on the basis of relatedness of the G glycoprotein (Anderson 1985, J. Infect. Dis. 151:626-633;
30 Mufson, et al., 1985, J. Gen. Virol. 66:2111-2124). The G protein of BRS virus strains included in these studies did not react with monoclonal antibodies generated against viruses from either HRS virus subgroup (Orvell et al., 1987, J. Gen. Virol. 68:3125-3135).

BRS virus provides an opportunity to study the role of the major glycoprotein, G, in attachment, the possible host range restrictions of BRS virus compared to HRS virus, and the roles of the individual viral antigens necessary to elicit a protective immune response in the natural host, which is something that cannot be done easily for HRS virus at present.

2.2. THE G PROTEIN

Previous work has shown that there is no cross reactivity between the attachment surface glycoproteins, G, of BRS virus and HRS virus, whereas there is cross antigenic reactivity between the other transmembrane glycoprotein, the fusion, F, protein and the major structural proteins, N, P, and M (Lerch et al., 1989, J. Virol. 63:833-840; Orville et al., 1987, J. Gen. Virol. 68:3125-3135). Available evidence indicates that BRS virus has a more narrow host restriction, infecting only cattle and bovine cells in culture, whereas HRS virus can infect a variety of cell types and experimental animals (Jacobs and Edington, 1975, Res. Vet. Sci. 18:299-306; Mohanty et al., 1976, J. Inf. Dis. 134:409-413; Paccaud and Jacquier, 1970, Arch. Gesamte Virusforsch 30:327-342). Since the G protein of HRS virus is the viral attachment protein (Levine et al., 1987, J. Gen. Virol. 68:2521-2524), this observation suggested that the differences in the BRS virus and HRS virus G proteins may be responsible for the differences in attachment and host range observed between BRS virus and HRS virus.

Based on sequence analysis of the HRS virus G mRNA, the G protein is proposed to have three domains; an internal or cytoplasmic domain, a transmembrane domain, and an external domain which comprises three quarters of the polypeptide (Satake et al., 1985, Nucl. Acids Res: 13:7795-7812; Wertz et al., 1985, Proc. Natl. Acad. Sci.

U.S.A. 82:4075-4079). Evidence suggests that the respiratory syncytial virus G protein is oriented with its amino terminus internal, and its carboxy terminus external, to the virion (Olmsted et al., 1989, J. Viral. 13:7795-7812; Vijaya et al., 1988, Mol. Cell. Biol. 8:1709-1714;
5 Wertz et al., 1985, Proc. Natl. Acad. Sci. U.S.A. 82:4075-4079). Unlike the other Paramyxovirus attachment proteins, the respiratory syncytial virus G protein lacks both neuraminidase and hemagglutinating activity (Gruber and
10 Levine, 1983, J. Gen. Virol. 64:825-832; Richman et al., 1971, Appl. Microbiol. 21:1099). The mature G protein, found in virions and infected cells, has an estimated molecular weight of 80-90kDa based on migration in SDS-
polyacrylamide gels (Dubovi, 1982, J. Virol. 42:372-378;
15 Gruber and Levine, 1983, J. Gen. Virol. 64:825-832; Lambert and Pons, 1983, Virology 130:204-214; Peebles and Levine, 1979, Virol. 95:137-145). In contrast, the G mRNA sequence predicts a protein with a molecular weight of 32kDa (Satake
20 et al., 1985, Nucl. Acids Res. 13:7795-7812; Wertz et al., 1985, Proc. Natl. Acad. Sci. U.S.A. 82:4075-4079), and when the G mRNA is translated in vitro it directs synthesis of a
36kDa protein that is specifically immunoprecipitated by anti-G serum. It has been shown that there is N-linked and
extensive O-linked glycosylation of the polypeptide backbone (Lambert, 1988, Virology 164:458-466; Wertz et
25 al., 1989, J. Virol. 63:X). Experiments using glycosidases (inhibitors of sugar addition) and a cell line defective in O-linked glycosylation suggest that 55% of the molecular
weight of the mature G protein is due to O-linked glycosylation, and 3% is due to N-linked glycosylation.
30 However, these estimates are based on migration in SDS-polyacrylamide gels and are only approximate values. Consistent with the evidence for extensive O-linked glycosylation is a high content (30%) of threonine and
35 serine residues in the predicted amino acid sequence of the

G protein (Satake et al., 1985, Nucl. Acids Res. 13:7795-7812; Wertz et al., 1985, Proc. Natl. Acad. Sci. U.S.A. 82:4075-4079). Threonine and serine are amino acid residues that serve as sites for O-linked oligosaccharide attachment (Kornfield and Kornfield, 1980, in "The
5 Biochemistry of Glycoproteins and Proteoglycans," Lenarz, ed., Plenum Press, N. Y. pp. 1-32) and in the HRS virus G protein 85% of the threonine and serine residues are in the extracellular domain (Wertz et al., 1985, Proc. Natl. Acad. Sci. U.S.A. 82:4075-4079). The high content of proline
10 (10%), serine and threonine (30%) and the extensive O-linked glycosylation of the G protein are features similar to those of a group of cellular glycoproteins known as the mucinous proteins (Ibid.), but unusual among viral
15 glycoproteins.

Isolates of HRS virus have been divided into two subgroups, A and B, based on the antigenic variation observed among G proteins using panels of monoclonal antibodies (Anderson et al., 1985, J. Inf. Dis. 151:626-633; Mufson et al., 1985, J. Gen. Virol. 66:2111-2124).
20 However, a few monoclonal antibodies exist which recognize the G protein of both subgroups (Mufson et al., 1985, J. Gen. Virol. 66:2111-2124; Orvell et al., 1987, J. Gen. Virol. 68:3125-3135). Sequence analysis of the G mRNA of
25 HRS viruses from the two subgroups showed a 54% overall amino acid identity between the predicted G proteins, with 44% amino acid identity in the extracellular domain of the protein (Johnson et al., 1987 Proc. Natl. Acad. Sci. U.S.A. 84:5625-5629).

30

2.3. THE F PROTEIN

The mature BRS virus F protein consists of two disulfide linked polypeptides, F₁ and F₂ (Lerch et al., 1989, J. Virol. 63:833-840). There are differences in the
35 electrophoretic mobility of the BRS virus and HRS virus F

protein in SDS-polyacrylamide gels (Lerch et al., 1989, supra). Polyclonal and most monoclonal antibodies react to the F protein of both HRS virus and BRS virus (Stott et al., 1984; Orvell et al., 1987; Kennedy et al., 1988, J. Gen. Virol. 69:3023-3032; Lerch et al., 1989, supra).

5 The fusion protein, F, of paramyxoviruses causes fusion of the virus to cells and fusion of infected cells to surrounding cells. Structurally, the F proteins of the various paramyxoviruses are similar to one another. The F protein is synthesized as a precursor, F₀, that is
10 proteolytically cleaved at an internal hydrophobic region to yield two polypeptides, F₁ and F₂, that are disulfide linked and form the active fusion protein. A carboxy terminal hydrophobic region in the F₁ polypeptide is
15 thought to anchor the F protein in the membrane with its carboxy terminus internal to the cell and the amino terminus external. The F protein is N-glycosylated (see review, Morrison, 1988, Virus Research 10:113-136). The HRS virus F protein is a typical paramyxovirus fusion
20 protein. Antibodies specific to the F protein will block the fusion of infected cells (Walsh and Hruska, 1983, J. Virol. 47:171-177; Wertz et al., 1987, J. Virol. 61:293-301) and also neutralize infectivity of the virus (Fernie and Gerin, 1982, Inf. Immun. 37:243-249; Walsh and Hruska,
25 1983, J. Virol. 47:171-177; Wertz et al., 1987, J. Virol. 61:293-301), but do not block attachment (Levine et al., 1987, J. Gen. Virol. 68:2521-2524). The F protein is synthesized as a polypeptide precursor F₀, that is cleaved into two polypeptides, F₁ and F₂. These two polypeptides
30 are disulfide linked and N-glycosylated (Fernie and Gerin, 1982, Inf. Immun. 37:243-249; Gruber and Levine, 1983, J. Gen. Virol. 64:825-832; Lambert and Pons, 1983, Virol. 130:204-24).

2.4. BOVINE RESPIRATORY SYNCYTIAL VIRUS VACCINES

Bovine respiratory syncytial virus (BRS) vaccines have been developed comprising live or inactivated virus, or viral proteins. Frennet et al. (1984, Ann. Med. Vet. 128:375-383) reported that 81 percent of calves administered a combined live BRS virus and bovine viral diarrhea vaccine were protected against severe respiratory symptoms induced by field challenge. Stott et al. (1984, J. Hyg. 93:251-262) compared an inactivated BRS viral vaccine (consisting of glutaraldehyde-fixed bovine nasal mucosa cells persistently infected with BRS virus and emulsified with oil adjuvant) to two live vaccines, one directed toward BRS virus and the other toward HRS virus. Eleven out of twelve calves given the inactivated viral vaccine in the Stott study (supra) were completely protected against BRS viral challenge, but all control animals and those given the live vaccines became infected.

It is possible that live vaccines may exacerbate BRS viral infection. A severe outbreak of respiratory disease associated with BRS virus occurred shortly after calves were vaccinated with a modified live BRS virus (Kimman et al., 1989, Vet. Q. 11:250-253). Park et al. (1989, Res. Rep. Rural Dev. Adm. 31:24-29) reports the development of a binary ethylenimine (BEI)-inactivated BRS virus vaccine which was tested for its immunogenicity in guinea pigs and goats. Serum neutralizing antibody was detected 2 weeks following inoculation and antibodies increased following a booster vaccination at four weeks. In goats, a protective effect against BRS virus was observed when animals were challenged with virus 12 weeks following inoculation.

Trudel et al. (1989, Vaccine 7:12-16) studied the ability of immunostimulating complexes, made from the surface proteins of both human (Long) and bovine (A-51908) RS strains, adsorbed to the adjuvant Quil A, to induce neutralizing antibodies. Immunostimulating complexes

prepared from bovine RS virus proteins were found to be significantly more efficient than their human counterpart in inducing neutralizing antibodies.

3. SUMMARY OF THE INVENTION

5 The present invention relates to recombinant DNA molecules which encode bovine respiratory syncytial (BRS) virus proteins, to BRS virus proteins and peptides and to recombinant BRS virus vaccines produced therefrom. It is based, in part, on the cloning of substantially full length
10 cDNAs which encode the entire BRS virus G, F, and N proteins. Nucleotide sequences of the G, F, and N cDNAs have been determined, and are set forth in Figure 2 (G protein), Figure 9 (F protein), and Figure 17 (N protein).

15 According to particular embodiments of the invention, DNA encoding a BRS virus protein or peptide may be used to diagnose BRS virus infection, or, alternatively, may be inserted into an expression vector, including, but not limited to, vaccinia virus, as well as bacterial, yeast,
20 insect, or other vertebrate vectors. These expression vectors may be utilized to produce the BRS virus protein or peptide in quantity; the resulting substantially pure viral peptide or protein may be incorporated into subunit vaccine formulations or may be used to generate monoclonal or
25 polyclonal antibodies which may be utilized in diagnosis of BRS virus infection or passive immunization. In additional embodiments, BRS virus protein sequence provided by the invention may be used to produce synthetic peptides or proteins which may be utilized in subunit vaccines, or
30 polyclonal or monoclonal antibody production. Alternatively, a nonpathogenic expression vector may itself be utilized as a recombinant virus vaccine.

4. DESCRIPTION OF THE FIGURES

Figure 1. Sequencing strategy of G cDNAs of BRS virus. The scale at the bottom indicates the number of nucleotides from the 5' end of the BRS virus G mRNA. cDNA were inserted into M13 mp19 replicative form (RF) DNA and the nucleotide sequence determined by dideoxynucleotide sequencing using a M13 specific sequencing primer. The arrow indicates the portion of the G mRNA sequence determined by extension of a synthetic oligonucleotide primer on BRS virus mRNA. The sequence of the primer was complementary to bases 154-166 of the BRS virus G mRNA sequence. The cDNAs in clones G4, G10 and G33 were also excised using PstI and KpnI and inserted into M13 mp18 RF DNA for sequencing of the opposite end of the cDNA. The lines for each clone indicate the sequence of the mRNA determined from that clone. Only clones G10 and G33 were sequenced in their entirety. Clones G1, G7, G12, G5 and G3 were all less than 500 nucleotides in length and only partially sequenced for this reason.

Figure 2. Alignment of the complete BRS virus G mRNA sequence with those of the HRS viruses A2 and 18537 G mRNAs. Alignment was done by the method of Needleman and Wunsch (1970, J. Mol. Biol. 48:443-453) comparing the BRS virus G sequence against the HRS virus A2 G sequence. Only nonidentical bases are shown for the G mRNA sequences of the HRS viruses A2 and 18537. Gaps, shown by the dotted lines, were used to maximize sequence identity of the HRS virus A2 G sequence as determined by Johnson et al. (1987, Proc. Natl. Acad. Sci. U.S.A. 84:5625-5629). The HRS virus consensus gene start and gene stop signals are overlined. The consensus initiation codon and the stop codon for the different viruses G mRNA are boxed. The dots above the sequences are spaced every ten nucleotides and the number of the last nucleotide on a line is indicated to the right of the sequence.

Figure 3. Alignment of the predicted amino acid sequence of the BRS virus G protein and the G proteins of the HRS viruses A2 and 18537. Alignment was done by the method of Needleman and Wunsch (1970, J. Mol. Biol. 48:443-453). Identical amino acids for the HRS virus A2 and 18537 G proteins are indicated by an asterisk. The proposed domains are indicated above the sequence. Potential N-linked carbohydrate addition sites are indicated for the BRS virus G protein by black triangles above the sequences, for the HRS virus A2 G protein by black diamonds above the sequences, and for the HRS virus 18537 protein by black triangles below the sequences. The four conserved cysteine residues are indicated by the dark circles. The conserved thirteen amino acid region of the HRS virus A2 and 18537 G proteins is boxed. A gap in the HRS virus A2 G protein sequence compared to the HRS virus 18537 G protein sequence, as described by Johnson et al. (1987, Proc. Natl. Acad. Sci. U.S.A. 84:5625-5629) is shown by a dash. The dots above the sequences are spaced every ten amino acid residues and the number of the last amino acid residue on a line is indicated to the right of the sequence.

Figure 4. Amino acid identity between the RS virus G proteins. The overall identity, and identity in the different proposed domains between the various G proteins is shown based on the alignment shown in Figure 3.

Figure 5. Hydrophilicity plots for the G protein of BRS virus, and the HRS viruses A2 and 18537. The distribution of the hydrophilic and hydrophobic regions along the predicted amino acid sequence of the G proteins was determined using the algorithm of Kyte and Doolittle (1982, J. Mol. Biol. 157:105-132). The value for each amino acid was calculated over a window of nine amino acids. The bottom scale indicates the amino acid residue.

starting with the amino terminal methionine. Hydrophilic regions of the amino acid sequence are shown above the axis, and hydrophobic regions below the axis.

Figure 6. Western blot analysis of BRS virus and recombinant vaccinia virus expressed G proteins. BT cells were infected with recombinant vaccinia viruses (moi=10), wild type vaccinia virus (moi=10) or BRS virus (moi=1). Proteins from BRS virus (lane B), wild type vaccinia virus (lane VV), recombinant vaccinia virus containing the BRS virus G gene in the forward (lane G642+) and reverse (lane G4567-) orientation, and mock (lane M) infected cells were harvested by lysing the cells at six hours postinfection for vaccinia virus infected cells, and 36 hours postinfection for BRS virus infected cells. The proteins were separated by electrophoresis in 10% polyacrylamide-SDS gel under non-reducing conditions, and analyzed by western blotting using anti-391-2 serum as a first antibody. Horseradish peroxidase-conjugated anti-bovine IgG was used to identify the bound first antibody. The BRS virus proteins are indicated. Prestained protein molecular weight markers are shown and labeled according to their molecular weight in kilodaltons.

Figure 7. Surface immunofluorescence of recombinant vaccinia virus infected cells. HEp-2 cells, grown on glass cover slips, were either mock (M) infected, infected with wild type vaccinia virus (VV; moi=10) or recombinant virus G642 (rVV; moi=10). At 24 hours postinfection, the live cells were stained with anti-391-2 serum, followed by fluorescein conjugated anti-bovine IgG (H+L). Phase contrast (left panels) and fluorescent (right panels) photographs are shown.

Figure 8. Sequencing strategy of cDNAs of BRS virus F messenger RNA. The scale at the bottom indicates the number of nucleotides from the 5' end of the BRS virus F mRNA. cDNAs were inserted into M13 mp19 replicative form

(RF) DNA and the nucleotide sequence was determined by dideoxynucleotide sequencing using a M13 specific sequencing primer. The lines for each clone indicate the portion of the mRNA sequence determined from that clone. The arrow indicates the area of the F mRNA sequence that was determined by extension of an oligonucleotide on BRS virus mRNA. The oligonucleotide was complementary to bases 267 to 284 of the BRS virus F mRNA. The cDNAs in clones F20, FB3, and FB5 were also excised using PstI and KpnI and inserted into M13 mp18 RF DNA for sequencing of the opposite end of the cDNA. Fragments of cDNAs in clones F20, FB5 and FB3 were generated by the use of the restriction enzymes EcoRI (FB3, FB5), or AlwNI (F20), PflMI (F20) and HpaI (F20) and EcoRV (F20) and subcloned back into M13 mp19, or mp18 RF DNA to determine the sequence in the internal areas of the F mRNA.

Figure 9. Nucleotide sequence of the BRS virus F mRNA. The nucleotide sequence of the BRS virus F mRNA as determined from cDNA clones and primer extension on BRS virus mRNA is shown. The dots above the sequence are spaced every ten nucleotides and the number of the last base on a line is indicated to the right of the sequence. The predicted amino acid sequence of the major open reading frame is also shown. The number of the last codon starting on a line is indicated to the right of the amino acid sequence. The potential N-linked glycosylation sites are boxed. The amino terminal and carboxy terminal hydrophobic domains are underlined in black as is the hydrophobic region at the proposed amino terminus of the F1 polypeptide. The proposed cleavage sequence is underlined in gray.

Figure 10. Hydrophilicity plot for the F protein of BRS virus. The distribution of the hydrophilic and hydrophobic regions along the predicted amino acid sequence of the F protein was determined using the algorithm of Kyte

and Doolittle (1982, J. Mol. Biol. 157:105-132). The value for each amino acid was calculated over a window of nine amino acids. The bottom scale indicates the amino acid residue starting with the amino terminal methionine.

Hydrophilic regions of the amino acid sequence are shown above the axis, and hydrophobic regions below the axis.

Figure 11. Alignment of the predicted amino acid sequences of the BRS virus F protein and the F proteins of HRS viruses A2, Long, RSS-2, and 18537. Alignment was done by the method of Needleman and Wunsch (1970, J. Mol. Biol. 48:443-453) comparing the BRS virus F protein against the F proteins of different HRS viruses. Only the non-identical amino acids are indicated for the HRS viruses F proteins. The dots below the sequence are spaced every ten amino acids and the number of the last residue on a line is indicated to the right of the sequence. The potential N-linked glycosylation sites of the proteins are boxed. Cysteine residues conserved between all five proteins are indicated by an open triangle. The amino terminal and carboxy terminal hydrophobic domains are underlined in black as is the hydrophobic region at the proposed amino terminus of the F1 polypeptide. The proposed cleavage sequence is underlined in gray.

Figure 12. Comparison of proteins from BRS virus and HRS virus infected cells synthesized in the presence and absence of tunicamycin. Proteins from BRS virus (B), HRS virus (H), or mock (M) infected cells were labeled by the incorporation of [³⁵S] methionine in the presence (lanes B_T, H_T, and M_T) or absence (lanes B, H and M) of tunicamycin. Virus specific proteins were immunoprecipitated using an anti-respiratory syncytial virus serum, and separated by electrophoresis on a 15% SDS-polyacrylamide gel. An autoradiograph of the gel is shown. All lanes are from the same gel with lanes H and HT

from a longer exposure than the other lanes. [^{14}C]-labeled protein molecular weight markers are shown and labeled according to their molecular weight in kilodaltons.

Figure 13. Expression of the BRS virus F protein from recombinant vaccinia virus infected cells. BT cells were
5 infected with recombinant vaccinia viruses (moi=10), wild type vaccinia virus (moi=10) or BRS virus (moi=1). Proteins from recombinant viruses containing the BRS virus F gene in the forward (lanes F464+) and reverse (F1597-) orientations and wild type vaccinia virus (VV) were
10 radioactively labeled by the incorporation of [^{35}S] methionine from three to six hours postinfection. Proteins from BRS virus infected (lane B), and mock infected cells (lane M) which were also radioactively labeled by the
15 incorporation of [^{35}S] methionine for three hours at 24 hours postinfection. The proteins were immunoprecipitated using the Wellcome anti-RS serum and compared by electrophoresis on a 15% polyacrylamide-SDS gel. Fluorography was done on the gel and an autoradiograph is
20 shown. The BRS virus F, N, M and P proteins are indicated. [^{14}C]-labeled protein molecular weight markers are shown and labeled according to their molecular weight in kilodaltons.

Figure 14. Comparison of the BRS virus and recombinant vaccinia virus expressed F proteins synthesized
25 in the presence of tunicamycin. BT cells were infected with recombinant vaccinia viruses (moi=10) wild type vaccinia virus (moi=10) or BRS virus (moi=1). Proteins from recombinant vaccinia viruses containing the BRS virus
30 F gene in the forward orientation (F 464=F), wild type vaccinia virus (V), BRS virus infected (B) and mock infected cells (M) were radioactively labeled by the incorporation of [^{35}S]-methionine for three hours, at three
hours postinfection for vaccinia infected cells and 24
35 hours postinfection for BRS virus infected cells, in the

absence (lanes F, V, B, M) or presence (lanes F_T, V_T, B_T, M_T) of tunicamycin. The proteins were immunoprecipitated using the Wellcome anti-RS serum and compared by electrophoresis on a 15% polyacrylamide-SDS gel.

5 Fluorography was done on the gel and an autoradiograph is shown. The BRS virus F, N, M and P proteins are indicated. [¹⁴C]-labeled protein molecular weight markers are shown and labeled according to their molecular weight in kilodaltons.

10 Figure 15. Surface immunofluorescence of recombinant vaccinia virus infected HEp-2 cells. HEp-2 cells, grown on glass cover slips, were either mock (M) infected, infected with wild type vaccinia virus (VV; moi=10), recombinant virus F 464 (rVVf; moi=10). At 24 hours postinfection, the
15 live cells were stained with anti-391-2 serum, followed by fluorescein conjugated anti-bovine IgG (H+L). Phase contrast (left panels) and fluorescent (right panels) photographs are shown.

Figure 16. Immunoprecipitation of ³H-glucosamine
20 labeled proteins from mock (M) bovine RS virus (Bov) or human RS virus (Hu) infected BT cells. Panel A. Antisera specific for the human RS virus G protein was prepared by immunizing rabbits with a recombinant VV vector (vG301) expressing the HRS virus A2 G protein (Stott et al., 1986,
25 J. Virol. 60:607-613) and used to immunoprecipitate the radiolabeled proteins from the mock, BRS virus (Bov) or HRS virus (Hu) infected cells. Panel B. Antisera specific for the bovine RS virus G protein was prepared by immunizing mice with a recombinant VV vector (vvG642)
30 expressing the BRS virus G and used to immunoprecipitate proteins from the mock, BRS virus (Bov) or HRS virus (Hu) infected cells. Panel C. Total ³H-glucosamine labeled proteins present in cytoplasmic extracts of mock, BRS virus (Bov) or HRS virus (Hu) infected cells.

Figure 17. Nucleotide sequence of the BRS virus N mRNA.

Figure 18. Amino acid sequence of BRS virus N protein.

5

10

15

20

25

30

35

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to bovine respiratory syncytial virus nucleic acids and proteins. For purposes of clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into the following subsections:

- (i) cloning bovine respiratory syncytial virus genes;
- (ii) expression of bovine respiratory syncytial virus proteins;
- (iii) the genes and proteins of the invention; and
- (iv) the utility of the invention.

5.1. CLONING BOVINE RESPIRATORY SYNCYTIAL VIRUS GENES

Bovine respiratory syncytial (BRS) virus genes, defined herein as nucleic acid sequences encoding BRS viral proteins, may be identified according to the invention by cloning cDNA transcripts of BRS virus mRNA and identifying clones containing full length BRS virus protein-encoding sequences, or, alternatively, by identifying BRS virus encoding nucleic acid sequences using probes derived from plasmids pRLG414-76-191, pRLF2012-76-192, or pRLNB3-76 or using oligonucleotide probes designed from the sequences presented in Figures 2, 9 or 17.

For example, and not by way of limitation, a cDNA containing the complete open reading frame of a BRS virus mRNA corresponding to a particular protein may be synthesized using BRS virus mRNA template and a specific synthetic oligonucleotide for second strand synthesis. The nucleotide sequence for the oligonucleotide may be determined from sequence analysis of BRS viral mRNA. First strand synthesis may be performed as described in D'Alessio et al. (1987, Focus 9:1-4). Following synthesis, the RNA template may be digested with RNase A (at least about

-19-

1 $\mu\text{g}/\mu\text{l}$) for about 30 minutes at 37 degrees Centigrade. The
resulting single-stranded cDNAs may then be isolated by
phenol extraction and ethanol precipitation. The
oligonucleotide used for second strand synthesis should
5 preferably have a sequence specific for the 5' end of the
viral mRNA of interest, and may also, preferably, contain a
useful restriction endonuclease cleavage site to facilitate
cloning. The single-stranded cDNAs may then be mixed with
the oligonucleotide (at about 50 $\mu\text{g}/\text{ml}$), heated to 100
10 degrees C for one minute and placed on ice. Reverse
transcriptase may then be used to synthesize the second
strand of the cDNAs in a reaction mixture which may
comprise 50 mM Tris-HCl (pH 8.0), 50 mM KCl, 5 mM MgCl_2 ,
10mM dithiothreitol, 1.6 mM dNTPs, and 100 U reverse
15 transcriptase, incubated for about one hour at 50 degrees
C. The cDNAs may then be separated from protein by phenol
extraction, recovered by ethanol precipitation, and ends
made blunt with T4 DNA polymerase. Blunt-ended cDNAs may
then be digested with an appropriate restriction
20 endonuclease (for example, which recognizes a cleavage site
in the oligonucleotide primer but not within the protein-
encoding sequence), separated from protein by phenol
extraction, and then cloned into a suitable vector.

Alternatively, cDNAs generated from viral mRNA may be
25 cloned into vectors, and clones carrying the nucleic acid
sequences of interest may be generated using, as probes,
oligonucleotides containing proteins of the nucleotide
sequences presented in Figures 2 and 9 for the BRS virus G
and F proteins, respectively. Viral cDNA libraries may be
30 screened, for example, using the method set forth in
Grunstein and Hogness (1975, Proc. Natl. Acad. Sci.
U.S.A.). Retrieved clones may then be analyzed by
restriction-fragment mapping and sequencing techniques
(e.g., Sanger et al., 1979, Proc. Natl. Acad. Sci. U.S.A.

72:3918-3921) well known in the art. Alternatively, all or portions of the desired gene may be synthesized chemically based on the sequence presented in Figure 2 or 9.

In additional embodiments, primers derived from the nucleic acid molecules of the invention and/or nucleic acid sequences as set forth in Figures 2 or 9, or nucleic acid sequences encoding amino acid sequences substantially as set forth in Figures 3 or 11, may be used in polymerase chain reaction (PCR); Saiki et al., 1985, Science 230:1350-1354) to produce nucleic acid molecules which encode BRS virus proteins or related peptides.

PCR requires sense strand as well as anti-sense strand primers. Accordingly, a degenerate oligonucleotide primer corresponding to one segment of BRS virus nucleic acid or amino acid sequence may be used as a primer for one DNA strand (e.g. the sense strand) and another degenerate oligonucleotide primer homologous to a second segment of BRS virus nucleic acid or amino acid sequence may be used as primer for the second DNA strand (e.g. the anti-sense strand). Preferably, these primers may be chosen based on a contiguous stretch of known amino acid sequence, so that the relevant DNA reaction product resulting from the use of these primers in PCR may be of a predictable size (i.e. the length of the product, in number of basepairs, should equal the sum of the lengths of the two primers plus three times the number of amino acid residues in the segment of protein bounded by the segments corresponding to the two primers). These primers may then be used in PCR with nucleic acid template which comprises BRS virus nucleic acid sequences, preferably cDNA prepared from BRS virus mRNA.

DNA reaction products may be cloned using any method known in the art. A large number of vector-host systems known in the art may be used. Possible vectors include, but are not limited to, cosmids, plasmids or modified viruses, but the vector system must be compatible with the

-21-

host cell used. Such vectors include, but are not limited to, bacteriophages such as lambda derivatives, or plasmids such as pBR322, pUC, or Bluescript® (Stratagene) plasmid derivatives. Recombinant molecules can be introduced into host cells via transformation, transfection, infection, electroporation, etc.

The BRS virus gene may be inserted into a cloning vector which can be used to transform, transfect, or infect appropriate host cells so that many copies of the gene sequences are generated. This can be accomplished by ligating the DNA fragment into a cloning vector which has complementary cohesive termini. However, if the complementary restriction sites used to fragment the DNA are not present in the cloning vector, the ends of the DNA molecules may be enzymatically modified. Alternatively, any site desired may be produced by ligating nucleotide sequences (linkers) onto the DNA termini; these ligated linkers may comprise specific chemically synthesized oligonucleotides encoding restriction endonuclease recognition sequences. Furthermore, primers used in PCR reactions may be engineered to comprise appropriate restriction endonuclease cleavage sites. In an alternative method, the cleaved vector and BRS viral gene may be modified by homopolymeric tailing. In specific embodiments, transformation of host cells with recombinant DNA molecules that incorporate an isolated BRS virus gene, cDNA, or synthesized DNA sequence enables generation of multiple copies of the gene. Thus, the gene may be obtained in large quantities by growing transformants, isolating the recombinant DNA molecules from the transformants and, when necessary, retrieving the inserted gene from the isolated recombinant DNA.

5.2. EXPRESSION OF BOVINE RESPIRATORY SYNCYTIAL VIRUS PROTEINS

The nucleotide sequence coding for a BRS virus protein, or a portion thereof, can be inserted into an appropriate expression vector, i.e., a vector which
5 contains the necessary elements for the transcription and translation of the inserted protein-coding sequence. The necessary transcriptional and translation signals can also be supplied by the native BRS viral gene. A variety of host-vector systems may be utilized to express the
10 protein-coding sequence. These include but are not limited to mammalian cell systems infected with virus (e.g., vaccinia virus, adenovirus, etc.); insect cell systems infected with virus (e.g., baculovirus); microorganisms such as yeast containing yeast vectors, or bacteria
15 transformed with bacteriophage DNA, plasmid DNA, or cosmid DNA. The expression elements of these vectors vary in their strengths and specificities. Depending on the host-vector system utilized, any one of a number of suitable transcription and translation elements may be used.

20 Any of the methods previously described for the insertion of DNA fragments into a vector may be used to construct expression vectors containing a chimeric gene consisting of appropriate transcriptional/translational control signals and the protein coding sequences. These
25 methods may include in vitro recombinant DNA and synthetic techniques and in vivo recombinations (genetic recombination). Expression of nucleic acid sequence encoding BRS virus protein or peptide fragment may be
30 regulated by a second nucleic acid sequence so that a BRS virus protein or peptide is expressed in a host transformed with the recombinant DNA molecule. For example, expression of BRS viral protein may be controlled by any
promoter/enhancer element known in the art. Promoters
35 which may be used to control BRS viral protein or peptide

expression include, but are not limited to, the CMV promoter (Stephens and Cockett, 1989, Nucl. Acids Res. 17:7110), the SV40 early promoter region (Bernoist and Chambon, 1981, Nature 290:304-310), the promoter contained in the 3' long terminal repeat of Rous sarcoma virus (Yamamoto, 5 et al., 1980, Cell 22:787-797), the herpes thymidine kinase promoter (Wagner et al., 1981, Proc. Natl. Acad. Sci. U.S.A. 78:144-1445), the regulatory sequences of the metallothionine gene (Brinster et al., 1982, Nature 296:39-42); prokaryotic expression vectors such as the β -lactamase promoter (Villa-10 Kamaroff, et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731), or the tac promoter (DeBoer, et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25), see also "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94; plant expression vectors comprising the15 nopaline synthetase promoter region (Herrera-Estrella et al., Nature 303:209-213) or the cauliflower mosaic virus 35S RNA promoter (Gardner, et al., 1981, Nucl. Acids Res. 9:2871), and the promoter for the photosynthetic enzyme ribulose20 biphosphate carboxylase (Herrera-Estrella et al., 1984, Nature 310:115-120); promoter elements from yeast or other fungi such as the Gal 4 promoter, the ADC (alcohol dehydrogenase) promoter, PGK (phosphoglycerol kinase) promoter, alkaline phosphatase promoter, and the following25 animal transcriptional control regions, which exhibit tissue specificity and have been utilized in transgenic animals: elastase I gene control region which is active in pancreatic acinar cells (Swift et al., 1984, Cell 38:639-646; Ornitz et al., 1986, Cold Spring Harbor Symp. Quant. Biol. 50:399-409;30 MacDonald, 1987, Hepatology 7:425-515); insulin gene control region which is active in pancreatic beta cells (Hanahan, 1985, Nature 315:115-122), immunoglobulin gene control region which is active in lymphoid cells (Grosschedl et al., 1984, Cell 38:647-658; Adames et al., 1985, Nature 318:533-538;35 Alexander et al., 1987, Mol. Cell. Biol. 7:1436-1444), mouse

mammary tumor virus control region which is active in testicular, breast, lymphoid and mast cells (Leder et al., 1986, Cell 45:485-495), albumin gene control region which is active in liver (Pinkert et al., 1987, Genes and Devel. 1:268-276), alpha-fetoprotein gene control region which is
5 active in liver (Krumlauf et al., 1985, Mol. Cell. Biol. 5:1639-1648; Hammer et al., 1987, Science 235:53-58); alpha 1-antitrypsin gene control region which is active in the liver (Kelsey et al., 1987, Genes and Devel. 1:161-171),
10 beta-globin gene control region which is active in myeloid cells (Mogram et al., 1985, Nature 315:338-340; Kollias et al., 1986, Cell 46:89-94; myelin basic protein gene control region which is active in oligodendrocyte cells in the brain (Readhead et al., 1987, Cell 48:703-712); myosin light
15 chain-2 gene control region which is active in skeletal muscle (Sani, 1985, Nature 314:283-286), and gonadotropic releasing hormone gene control region which is active in the hypothalamus (Mason et al., 1986, Science 234:1372-1378).

Expression vectors containing BRS virus gene inserts
20 can be identified by three general approaches: (a) DNA-DNA hybridization, (b) presence or absence of "marker" gene functions, and (c) expression of inserted sequences. In the first approach, the presence of a foreign gene inserted in an expression vector can be detected by DNA-DNA hybridization
25 using probes comprising sequences that are homologous to an inserted BRS virus gene. In the second approach, the recombinant vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (e.g., thymidine kinase activity, resistance to
30 antibiotics, transformation phenotype, occlusion body formation in baculovirus, etc.) caused by the insertion of foreign genes in the vector. For example, if the BRS gene is inserted within the marker gene sequence of the vector, recombinants containing the BRS insert can be identified by
35 the absence of the marker gene function. In the third

approach, recombinant expression vectors can be identified by assaying the foreign gene product expressed by the recombinant.

Once a particular recombinant DNA molecule is identified and isolated, several methods known in the art may be used to propagate it. Once a suitable host system and growth conditions are established, recombinant expression vectors can be propagated and prepared in quantity. As previously explained, the expression vectors which can be used include, but are not limited to, the following vectors or their derivatives: human or animal viruses such as vaccinia virus or adenovirus, in particular bovine adenovirus, as well as bovine herpes virus; insect viruses such as baculovirus; yeast vectors; bacteriophage vectors (e.g., lambda), and plasmid and cosmid DNA vectors, to name but a few.

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or modifies and processes the gene product in the specific fashion desired. Expression from certain promoters can be elevated in the presence of certain inducers; thus, expression of the genetically engineered BRS virus protein may be controlled. Furthermore, different host cells have characteristic and specific mechanisms for the translational and post-translational processing and modification (e.g., glycosylation, cleavage) of proteins. Appropriate cell lines or host systems can be chosen to ensure the desired modification and processing of the foreign protein expressed. For example, expression in a bacterial system can be used to produce an unglycosylated core protein product. Expression in mammalian cells can be used to ensure "native" glycosylation of the heterologous BRS virus protein. Furthermore, different vector/host expression systems may effect processing reactions such as proteolytic cleavages to different extents.

Once a recombinant which expresses the BRS virus gene is identified, the gene product should be analyzed. This can be achieved by assays based on the physical or functional properties of the product.

5 Once the BRS virus protein or peptide is identified, it may be isolated and purified by standard methods including chromatography (e.g., ion exchange, affinity, and sizing column chromatography), centrifugation, differential solubility, or by any other standard technique for the purification of proteins.

10 In additional embodiments of the invention, BRS virus proteins or peptides may be produced by chemical synthesis using methods well known in the art. Such methods, are reviewed, for example, in Barany and Merrifield (1980, in "The Peptides," Vol. II, Gross and Meienhofer, eds., J. Academic Press, N.Y. pp. 1-284)

15 Additionally, recombinant viruses which infect but are nonpathogenic in cattle, including but not limited to vaccinia virus, bovine herpes virus and bovine adenovirus, may be engineered to express BRS virus proteins using
20 suitable promoter elements. Such recombinant viruses may be used to infect cattle and thereby produce immunity to BRS virus. Additionally, recombinant viruses capable of expressing BRS virus protein, but which are potentially pathogenic, may be inactivated prior to administration as a
25 component of a vaccine.

5.3. THE GENES AND PROTEINS OF THE INVENTION

Using the methods detailed supra and in Example
30 Sections 6 and 7 infra, the following nucleic acid sequences were determined, and their corresponding amino acid sequences deduced. The BRS virus G protein cDNA sequence was determined, and the corresponding mRNA sequence is depicted in FIG. 2. BRS virus F protein cDNA sequence was determined,
35 and the corresponding mRNA sequence is depicted in FIG. 9.

BRS virus N protein cDNA sequence was determined, and the corresponding sequence is depicted in Figure 17. Each of these sequences, or their functional equivalents, can be used in accordance with the invention. The invention is further directed to sequences and subsequences of BRS virus G, F or N protein encoding nucleic acids comprising at least ten nucleotides, such subsequences comprising hybridizable portions of the BRS virus G, F or N encoding nucleic acid sequence which have use, e.g., in nucleic acid hybridization assays, Southern and Northern blot analyses, etc. The invention also provides for BRS virus G, F or N proteins, fragments and derivatives thereof, according to the amino acid sequences set forth in FIGS. 3, 11 or 18 or their functional equivalents or as encoded by the following cDNA clones as deposited with the ATCC: pRLG414-76-191, pRLF2012-76-192, pRLNB3-76. The invention also provides fragments or derivatives of BRS virus G or F proteins which comprise antigenic determinant(s).

For example, the nucleic acid sequences depicted in Figures 2, 9 or 17 can be altered by substitutions, additions or deletions that provide for functionally equivalent molecules. Due to the degeneracy of nucleotide coding sequences, other DNA sequences which encode substantially the same amino acid sequence as depicted in FIGS. 3, 11 or 18 may be used in the practice of the present invention. These include but are not limited to nucleotide sequences comprising all or portions of the nucleotide sequences encoding BRS virus G, F or N proteins depicted in FIGS. 2, 9 or 18 which are altered by the substitution of different codons that encode a functionally equivalent amino acid residue within the sequence, thus producing a silent change. Likewise, the BRS virus G, F or N proteins, or fragments or derivatives thereof, of the invention include, but are not limited to, those containing, as a primary amino acid sequence, all or part of the amino acid sequence

substantially as depicted in FIGS. 3, 11 or 18 or as encoded by pRLG414-76-191, pRLF2012-76-192, or pRLNB3-76 including altered sequences in which functionally equivalent amino acid residues are substituted for residues within the sequence resulting in a silent change. For example, one or more amino acid residues within the sequence can be substituted by another amino acid of a similar polarity which acts as a functional equivalent, resulting in a silent alteration. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Also included within the scope of the invention are BRS virus proteins or fragments or derivatives thereof which are differentially modified during or after translation, e.g., by glycosylation, proteolytic cleavage, linkage to an antibody molecule or other cellular ligand, etc.

In addition, the recombinant BRS virus G, F or N protein encoding nucleic acid sequences of the invention may be engineered so as to modify processing or expression of BRS virus protein. For example, and not by way of limitation, a signal sequence may be inserted upstream of BRS virus G, F or N protein encoding sequences to permit secretion of BRS virus G, F or N protein and thereby facilitate harvesting or bioavailability.

Additionally, a given BRS virus protein-encoding nucleic acid sequence can be mutated in vitro or in vivo, to create and/or destroy translation, initiation, and/or termination sequences, or to create variations in coding

regions and/or form new restriction endonuclease sites or destroy preexisting ones, to facilitate further in vitro modification. Any technique for mutagenesis known in the art can be used, including but not limited to, in vitro site-directed mutagenesis (Hutchinson, et al., 1978, J. Biol. Chem. 253:6551), use of TAB® linkers (Pharmacia), etc.

5.4. THE UTILITY OF THE INVENTION

5.4.1. BOVINE RESPIRATORY SYNCYTIAL VIRUS VACCINES

10 The present invention may be utilized to produce safe and effective BRS virus vaccines. According to the invention, the term vaccine is construed to refer to a preparation which elicits an immune response directed toward a component of the preparation. Advantages of the present invention include the capability of producing BRS
15 virus proteins in quantity for use in vaccines or for the generation of antibodies to be used in passive immunization protocols as well as the ability to provide a recombinant virus vaccine which produces immunogenic BRS virus proteins but which is nonpathogenic. These alternatives circumvent
20 the use of modified live BRS virus vaccines which may cause exacerbated symptoms in cattle previously exposed to BRS virus.

According to the invention, the BRS virus nucleic acid
25 sequences may be inserted into an appropriate expression system such that a desired BRS virus protein is produced in quantity. In specific embodiments of the invention, BRS virus G, F or N proteins may be produced in quantity in this manner for use in subunit vaccines. In preferred
30 specific embodiments of the invention, the BRS virus G, F or N protein may be expressed by recombinant vaccinia viruses, including, but not limited to, rVVF464 (F protein producing virus), or rVVG642 (G protein producing virus), harvested, and then administered as a protein subunit in a
35 suitable pharmaceutical carrier.

Alternatively, recombinant virus, including, but not limited to, vaccinia virus, bovine herpes virus and bovine adenovirus and retroviruses which are nonpathogenic in cattle but which express BRS virus proteins, may be used to infect cattle and thereby produce immunity to BRS virus without associated disease. The production of the BRS G protein in recombinant virus vaccinated animals, or a portion or derivative thereof, may additionally prevent attachment of virus to cells, and thereby limit infection.

In further embodiments of the invention, BRS virus protein or peptide may be chemically synthesized for use in vaccines.

In vaccines which comprise peptide fragments of a BRS virus protein, it may be desirable to select peptides which are more likely to elicit an immune response. For example, the amino acid sequence of a BRS virus protein may be subjected to computer analysis to identify surface epitopes using a method such as, but not limited to, that described in Hopp and Woods (1981, Proc. Natl. Acad. Sci. U.S.A. 78:3824-3828), which has been successfully used to identify antigenic peptides of Hepatitis B surface antigen. It may also be desirable to modify the BRS virus peptides in order to increase their immunogenicity, for example, by chemically modifying the peptides or by linking the peptides to a carrier molecule.

The vaccines of the invention may be administered, in a suitable pharmaceutical carrier, by a variety of routes, including, but not limited to, nasally, orally, intramuscularly, subcutaneously, or intravenously and preferably intratracheally or by scarification. In preferred embodiments of the invention, between about 10^6 and 10^8 recombinant viruses may be administered in an inoculation. It may be desirable to administer subunit vaccines of the invention together with an adjuvant, for example, but not limited to, Freund's (complete or

-31-

incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanins, dinitrophenol, or Bacille calmette-Guerin (BCG) or Corynebacterium parvum. Multiple
5 inoculations may be necessary in order to achieve protective and/or long-lasting immunity.

5.4.2. ANTIBODIES DIRECTED TOWARD BOVINE RESPIRATORY SYNCYTIAL VIRUS PROTEINS

10 In additional embodiments, nucleic acid, protein or peptide molecules of the invention may be utilized to develop monoclonal or polyclonal antibodies directed toward BRS virus protein. For preparation of monoclonal antibodies directed toward a BRS virus protein, any
15 technique which provides for the production of antibody molecules by continuous cell lines in culture may be used. For example, the hybridoma technique originally developed by Kohler and Milstein (1975, Nature 256:495-497) may be used.

20 A molecular clone of an antibody to a BRS virus protein may be prepared by known techniques. Recombinant DNA methodology (see e.g. Maniatis et al., 1982, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York) may be used to
25 construct nucleic acid sequences which encode a monoclonal antibody molecule or antigen binding region thereof.

Antibody molecules may be purified by known techniques, e.g., immunoabsorption or immunoaffinity chromatography, chromatographic methods such as HPLC (high
30 performance liquid chromatography), or a combination thereof, etc.

Antibody fragments which contain the idiotype of the molecule can be generated by known techniques. For
35 example, such fragments include but are not limited to: the

F(ab')₂ fragment which can be produced by pepsin digestion of the antibody molecule; the Fab' fragments which can be generated by reducing the disulfide bridges of the F(ab')₂ fragment, and the 2 Fab or Fab fragments which can be generated by treating the antibody molecule with papain and a reducing agent.

5.4.3. DIAGNOSTIC APPLICATIONS

The present invention, which relates to nucleic acids encoding BRS virus proteins and to proteins, peptide
10 fragments, or derivatives produced therefrom, as well as antibodies directed against BRS virus proteins and peptides, may be utilized to diagnose BRS virus infection.

For example, the nucleic acid molecules of the
15 invention may be utilized to detect the presence of BRS viral nucleic acid in BRS virus-infected animals by hybridization techniques, including Northern blot analysis, wherein a nucleic acid preparation obtained from an animal is exposed to a potentially complementary nucleic acid
20 molecule of the invention under conditions which allow hybridization to occur and such that hybridization may be detected. For example, and not by way of limitation, total RNA may be prepared from swabs of nasal tissue, tracheal swabs, or isolates from any upper respiratory tract mucosal
25 surface obtained from a cow potentially infected with BRS virus. The RNA may then be subjected to Northern blot analysis in which detectably labeled (e.g. radiolabeled) oligonucleotide probe derived from a BRS virus nucleic acid may be exposed to a Northern blot filter bearing the cow
30 RNA under conditions permissive for hybridization; following hybridization, the filter may be washed and binding of probe to the filter may be visualized by autoradiography. Alternatively, if low levels of BRS virus are present in a diagnostic sample, it may be desirable to
35 detect the presence of BRS virus nucleic acid using

oligonucleotides of the invention in PCR reaction in order to amplify the amount of BRS virus nucleic acid present. In a preferred embodiment of the invention, viral RNA amplified from cultured cells may be analyzed for RS virus sequences by dot blot hybridization. The presence of BRS
5 virus sequence not provided by the primer in the product of such a PCR reaction would be indicative of BRS virus infection.

In further embodiments, the BRS virus proteins and peptides of the invention may be used in the diagnosis of
10 BRS virus infection. For example, and not by way of limitation, BRS virus proteins and peptides may be utilized in enzyme linked immunosorbent assay (ELISA), immunoprecipitation, rosetting or Western blot techniques to detect the presence of anti-BRS virus antibody. In
15 preferred embodiments, a rise in the titer of anti-BRS virus antibodies may be indicative of active BRS virus infection. According to these embodiments, a serum sample may be exposed to BRS virus protein or peptide under
20 conditions permissive for binding of antibody to protein or peptide and such that binding of antibody to protein or peptide may be detected. For example, and not by way of limitation, BRS virus protein or peptide may be immobilized on a solid surface, exposed to serum potentially comprising
25 anti-BRS virus antibody (test serum) under conditions permissive for binding of antibody to protein or peptide, and then exposed to an agent which permits detection of binding of antibody to BRS virus protein or peptide, e.g. a detectably labeled anti-immunoglobulin antibody.

30 Alternatively, BRS virus protein or peptide may be subjected to Western blot analysis, and then the Western blot may be exposed to the test serum, and binding of antibody to BRS virus protein or peptide may be detected as set forth above. In further, non-limiting embodiments of
35 the invention, BRS virus protein or peptide may be adsorbed

onto the surface of a red blood cell, and such antigen-coated red blood cells may be exposed to serum which potentially contains anti-BRS virus antibody. Rosette formation by serum of antigen coated red blood cells may be indicative of BRS virus exposure or active infection.

5 In additional embodiments of the invention, antibodies which recognize BRS virus proteins may be utilized in ELISA or Western blot techniques in order to detect the presence of BRS virus proteins, which would be indicative of active BRS virus infection.

10

6. EXAMPLE: NUCLEOTIDE SEQUENCE OF THE ATTACHMENT PROTEIN, G, OF BOVINE RESPIRATORY SYNCYTIAL VIRUS AND EXPRESSION FROM A VACCINIA VIRUS VECTOR

6.1. MATERIALS AND METHODS

15

6.1.1. VIRUS AND CELLS

The growth and propagation of BRS virus isolate 391-2, wild type (Copenhagen strain) and recombinant vaccinia viruses (VV) bovine nasal turbinate (BT) cells, HEP-2 cells, and thymidine kinase negative (tk⁻) 143B cells were as described in Hruby and Ball (1981, J. Virol. 40:456-464), Lerch et al., (1989, J. Virol. 63:833-840) and Stott et al. (1986, J. Virol. 60:607-613).

25

6.1.2. CDNA SYNTHESIS, MOLECULAR CLONING, AND IDENTIFICATION OF BRS VIRUS G SPECIFIC CDNA CLONES

30 cDNAs were synthesized using the strand replacement method of Gubler and Hoffman (1983, Gene 25:263-269) as described in D'Allesio, et al. (1987, Focus 9:1-4). T4 DNA polymerase (BRL) was used to make the ends of the cDNAs blunt (Maniatis et al., 1982, in "Molecular Cloning, a laboratory manual", Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). The cDNAs were ligated into M13mp19 replicative form (RF) DNA, which had been digested with SmaI and treated with calf intestinal alkaline phosphatase

35

-35-

and transfected into competent E. coli DH5 α F' cells (Bethesda Research Laboratories; Hanahan, 1983, J. Mol. Biol. 166:557-580). M13mp19 phage containing BRS virus G specific inserts were identified by dot blot hybridization of phage DNA (Davis, et al., 1986, in "Basic Methods in
5 Molecular Biology," Elsevier Science Publishing Co., Inc., NY, NY) probed with a BRS virus G clone (Lerch et al., 1989, J. Virol. 63:833-840) that had been labeled by nick translation (Rigby, et al., 1977, J. Mol. Biol. 113:237-
10 251). Growth and manipulations of M13mp19 and recombinant phage were as described by Messing (1983, Methods Enzymol. 101:20-78).

6.1.3. NUCLEOTIDE SEQUENCING AND PRIMER EXTENSION ON RNA

15 Dideoxynucleotide sequencing using the Klenow fragment of E. coli DNA polymerase I (Pharmacia) or a modified T7 DNA polymerase (Sequenase, U.S. Biochemicals) was done essentially as described in Lim and Pene (1988, Gene Anal. Tech. 5:32-39) and Tabor and Richardson (1987, Proc. Natl.
20 Acad. Sci. U.S.A. 84:4746-4771) using an M13 sequencing primer (New England Biolabs). Extension of a synthetic DNA primer, complementary to bases 154 to 166 of the BRS virus G mRNA, on BRS virus mRNA template using Avian
myeloblastosis virus (AMV) reverse transcriptase (Molecular
25 Genetic Resources) was done to determine the 5' sequence of the BRS virus G mRNA (Air, 1979, Virol. 97:468-472). BRS virus mRNA used as template in the primer extension on RNA was harvested as described for mRNA used for cDNA synthesis (Lerch et al., 1989, J. Virol. 63:833-840). Nucleotide
30 sequencing and primer extension were done using [α -³⁵S] dATP (Amersham) and polyacrylamide-urea gradient gel electrophoresis as described by Biggin et al. (1983, Proc. Natl. Acad. Sci. U.S.A. 80:3963-3965). The nucleotide

sequence was analyzed using the University of Wisconsin Genetics Computer Group software package (Devereux, et al., 1984, Nucleic Acids Res. 12:387-395).

5 6.1.4. SYNTHESIS AND CLONING OF COMPLETE cDNAs
 TO THE BRS VIRUS G mRNA

A cDNA containing the complete open reading frame of the BRS virus G mRNA was synthesized using a specific synthetic oligonucleotide for second strand synthesis. The nucleotide sequence for the oligonucleotide was determined
10 from sequence analysis of BRS virus mRNAs. First strand synthesis occurred as described in D'Alessio et al. (1987, Focus 9:1-4). Following synthesis, the RNA template was digested with RNase A (1 μ g/ μ l) for thirty minutes at 37 degrees Centigrade (C). The resulting single stranded
15 cDNAs were isolated by phenol extraction and ethanol precipitation. The oligonucleotide used for second strand synthesis had the sequence 5'CACGGATCCACAAGTATGTCCAACC 3' with the 5' first nine bases of the oligonucleotide containing a BamHI restriction enzyme site in the gene.
20 The single-stranded cDNAs were mixed with the oligonucleotides at a concentration of about 50 μ g/ml, heated to 100 degrees C for one minute and placed on ice. AMV reverse transcriptase was used to synthesize the second strand of the cDNAs in a reaction comprising 50 mM Tris-HCl
25 (pH8.0), 50 mM KCl, 5mM MgCl₂, 10mM dithiothreitol, 1.6mM dNTPs, and 100 U AMV reverse transcriptase] that was incubated for one hour at 50°C. The cDNAs were separated from protein by phenol extraction, recovered by ethanol precipitation, and ends made blunt with T4 DNA polymerase.
30 Blunt-ended cDNAs were then digested with the restriction enzyme BamHI (Bethesda Research Laboratories) and separated from protein by phenol extraction. M13mpl8 RF DNA, that was digested with BamHI and SmaI and treated with calf
35 intestinal phosphatase, was mixed in solution with the

-37-

CDNAs and recovered by ethanol precipitation. The cDNAs and vector were ligated and transfected into competent DH5 α F' cells (Bethesda Research Laboratories) as described in Hanahan (1983, J. Mol. Biol. 166:557-580).

5

6.1.5. CONSTRUCTION AND ISOLATION OF RECOMBINANT VACCINIA VIRUS VECTORS

A complete cDNA clone, G4, corresponding to the BRS virus G mRNA was subcloned into a recombinant plasmid, pIB176-192, by digestion with BamHI and KpnI, treatment
10 with T4 DNA polymerase to make the ends of the cDNA blunt, and ligation into the unique SmaI site of pIB176-192. The plasmid pIB176-192 is similar to recombination plasmids described in Ball, et al, (1986, Proc. Natl. Acad. Sci. USA 83:246-250). pIB176-192 contains base pairs (bp) 1 to 1710
15 of the HindIII J fragment of vaccinia virus inserted between the HindIII and SmaI sites of pIB176 (International Biotechnology Inc.). Inserted into the EcoRI site (bp 670 of the HindIII J fragment) of the vaccinia virus thymidine kinase (tk) gene (bp 502 to 1070 of the HindIII J fragment;
20 Weir and Moss, 1983, J. Virol. 46: 530-537) is a 280bp fragment of DNA that contains the 7.5K promoter of vaccinia virus. The orientation of this promoter fragment was such that it directed transcription from right to left on the conventional vaccinia virus map, opposite to the direction
25 of transcription of the vaccinia virus tk gene. The unique SmaI site in pIB176-192 is downstream from the major transcriptional start site of the 7.5K promoter.

The isolation of recombinant vaccinia viruses containing the BRS virus G mRNA sequence was as described
30 in Stott, et al. (1986, J. Virol. 60:607-613) except that the recombinant viruses were identified using the blot procedure of Lavi and Ektin (1981, Carcinogenesis 2:417-423).

35

6.1.6. CHARACTERIZATION OF RECOMBINANT VACCINIA VIRUS VECTORS

Vaccinia virus core DNA from wild type and recombinant viruses was prepared for Southern blot analysis as described by Esposito et al. (1981, J. Virol. Methods 2:175-179). Restriction enzyme digestion, Southern blot analysis, and radioactive labeling of DNA by nick translation were performed using standard techniques (Maniatis et al., 1982, in "Molecular Cloning: a laboratory manual," Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.; Rigby, et al., 1977, J. Mol. Biol. 113:237-251). Metabolic labeling of proteins from BRS virus and wild type and recombinant vaccinia virus infected cells was as described in Lerch et al. (1989, J. Virol. 63:833-840) except that proteins in vaccinia virus infected cells were labeled for three hours starting at three hours postinfection. SDS-polyacrylamide gel electrophoresis of proteins was done using standard procedures. For western blot analysis, proteins from infected and uninfected cells were harvested as described in Lerch et al. (1989, J. Virol. 63:833-840) at thirty hours postinfection for BRS virus infected BT cells, and six hours postinfection for vaccinia virus infected BT cells. Anti-BRS virus 391-2 serum (*ibid*) was used in the western blot analysis. Immunofluorescence was done on HEp-2 grown on glass cover slips. Hep-2 cells were infected with wild type or recombinant vaccinia viruses (multiplicity of infection (moi)=10). At 48 hours postinfection, the cells were stained for surface immunofluorescence using anti-BRS virus 391-2 serum as a first antibody followed by fluorescein conjugated anti-bovine IgG (H+L). Fluorescence was observed through a Nikon fluorescence microscope.

6.2. RESULTS

6.2.1. NUCLEIC ACID SEQUENCE AND COMPARISON

In order to determine the sequence of the BRS virus G mRNA and deduce the amino acid sequence, cDNAs to the BRS virus G mRNA were generated, and the nucleotide sequence was determined from nine cDNA clones. The nine clones were derived independently from four separate cDNA synthesis reactions. Direct sequencing of the 5' end of G mRNA by primer extension of a synthetic DNA primer on BRS virus mRNA was also performed. The areas of the BRS virus G mRNA sequence determined from the different clones and from primer extension are shown in Figure 1. Clone G4 is a full length BRS virus G clone synthesized using an oligonucleotide primer specific for second strand synthesis. Three independent clones, G4 (bases 8-838), G10 (bases 19-828) and G33 (bases 23-808), were excised with PstI and KpnI, and subcloned into M13mp18 RF DNA to allow for sequencing from the opposite end of the cDNA. Clones G10 and G33 were sequenced in their entirety. Clones G1, G7, G12, G5, and G3 were all less than 500 nucleotides in length and only partially sequenced for this reason.

The BRS virus G protein mRNA was found to be about 838 nucleotides in length excluding the polyadenylate tail (Figure 2). The BRS virus G mRNA sequence was compared to the published nucleotide sequences of the G protein mRNAs of HRS viruses A2 and 18537, a subgroup A virus and a subgroup B virus, respectively (Figure 2) (Johnson et al., 1987, Proc. Natl. Acad. Sci. U.S.A. 84:5625-5629; Wertz et al., 1985, Proc. Natl. Acad. Sci. U.S.A. 82: 4075-4079).

The BRS virus G mRNA was shorter than the HRS virus A2 and 18537 G mRNAs by 81 and 84 bases, respectively. There were some conserved features in common between the BRS virus G mRNA and the HRS virus G mRNAs. With the exception of the first nucleotide, the BRS virus G mRNA had the conserved

gene start signal 5'GGGGCAAU...3'. Collins, et al., (1986, Proc. Natl. Acad. Sci. USA 83:4594-4598) found at the 5' end of all HRS virus mRNAs. The 3' end of the BRS virus G mRNA also conformed to one of the two consensus gene end sequences 5'...AGU^AAU^AUpoly A3' found at the 3' end of all HRS virus genes (Collins, et al., 1986, Proc. Natl. Acad. Sci. USA 83:4594-4598). The position of the initiation codon, nucleotides 16-18, for the major open reading frame of the BRS virus G mRNA was identical to the position of the initiation codons of the G mRNAs of HRS viruses (Johnson et al., 1987, Proc. Natl. Acad. Sci. U.S.A. 84:5625-5629; Satake, et al., 1985, Nucleic Acids Res. 13:7795-7812; Wertz et al., 1985, Proc. Natl. Acad. Sci. U.S.A. 82:4075-4079). The noncoding region at the 3' end of the BRS virus mRNA, however, was 42 bases long compared to six bases for the HRS virus A2G mRNA and 27 bases for the HRS virus 18537 G mRNA (Johnson et al., 1987, Proc. Natl. Acad. Sci. U.S.A. 84:5625-5629; Satake, et al., 1985, Nucleic Acids Res. 13:7795-7812; Wertz et al., 1985, Proc. Natl. Acad. Sci. U.S.A. 82:4075-4079). This resulted in the termination codon for the BRS virus G protein occurring 119 bases prior to the termination codon for the G protein of HRS virus A2, and 98 bases prior to the termination codon in the 18537 G mRNA. One clone, G4, was missing bases 812, 819 and 824, all of which were after the termination codon in the 3' noncoding region. The BRS virus G mRNA lacked an upstream ATG found in the HRS virus G mRNAs (Johnson et al., 1987, Proc. Natl. Acad. Sci. U.S.A. 84:5625-5629; Satake, et al., 1985, Nucleic Acids Res. 13:7795-7812; Wertz et. al., 1985, Proc. Natl. Acad. Sci. U.S.A. 82:4075-4079).

The BRS virus G mRNA shared 51.7% sequence identity with the HRS virus A2 G mRNA and 50.8% with the HRS virus 18537 G mRNA when the BRS virus G mRNA was aligned with the HRS virus A2 G mRNA. The HRS virus A2 and 18537 G mRNAs

share 67.4% sequence identity (Johnson et al., 1987, Proc. natl. Acad. Sci. U.S.A. 84: 5625-5629). A slightly different alignment occurs when the BRS virus G mRNA is aligned with the HRS virus 18537 G mRNA, but resulted in a change of less than 1% in sequence identity between the G mRNA of BRS virus and the G mRNA of either HRS virus A2 or 18537 viruses. Computer analysis was used to determine if an internal deletion would result in a better alignment, but one was not found.

10

6.2.2. AMINO ACID SEQUENCE AND COMPARISON

The BRS virus G mRNA had a major open reading frame which predicted a polypeptide of 257 amino acids. The predicted molecular weight of this polypeptide was 28.6kD. The deduced amino acid sequence of the BRS virus G protein is shown (Figure 3) and compared with the published amino acid sequences of the HRS virus A2 and 18537 G proteins (Johnson et al., 1987, Proc. Natl. Acad. Sci. U.S.A. 84:5625-5629; Wertz et al., 1985, Proc. Natl. Acad. Sci. U.S.A. 82:4075-4079). The BRS virus G protein was similar in overall amino acid composition to the HRS virus G proteins, with a high content of threonine and serine residues, 25.7%, compared to that observed for the G proteins of the HRS viruses 18537 (28.4%) and A2 (30.6%). Serine and threonine residues are potential sites for the addition of O-linked carbohydrate side chains. Out of 66 threonine and serine residues in the BRS virus protein, 52 (79%) of these are in the proposed extracellular domain. The position of only nine threonine residues (amino acids 12, 52, 72, 92, 129, 139, 199, 210, 211, 235) and eight (8) serine residues (amino acids 2, 28, 37, 44, 53, 102, 109, 174) were conserved between predicted amino acid sequences of all the G proteins examined to date (Figure 3). In addition to the potential O-linked carbohydrate addition sites, there were four sites for potential N-linked

carbohydrate addition in the BRS virus G protein. The position of none of the potential N-linked addition sites was conserved between BRS virus and the subtype B HRS virus; two of the four potential sites were the same in HRS virus A2 and BRS virus G proteins.

5 The BRS virus G protein had a high proline content, 7.8%, similar to that observed for the G proteins of the HRS viruses A2 (10%) and 18537 (8.6%) (Johnson et al., 1987, Proc. Natl. Acad. Sci. U.S.A. 84:5625-5629; Satake, et al., 1985, Nucleic Acids Res. 13:7795-7812; Wertz et
10 al., 1985, Proc. Natl. Acad. Sci. U.S.A. 82:4075-4079). Six proline residues were conserved among all RS virus G proteins sequenced to date. These proline residues were amino acids 146, 155, 156, 172, 194, and 206 (Figure 3).

15 There were four cysteine residues in the proposed extracellular domain of the BRS virus G protein. These four residues were exactly conserved in position, relative to the amino terminus of the protein, with the four cysteine residues conserved among the HRS virus A2, Long
20 and 18537 (Figure 3) (Johnson et al., 1987, Proc. Natl. Acad. Sci. U.S.A. 84:5625-5629; Satake, et al., 1985, Nucleic Acids Res. 13:7795-7812; Wertz et al., 1985, Proc. Natl. Acad. Sci. U.S.A. 82:4075-4079). In addition, the G
25 proteins of BRS virus, HRS virus A2, and HRS virus 18537 all shared a cysteine residue in the proposed cytoplasmic domain (Figure 3). However, this cysteine residue is not present in the HRS virus Long G protein.

While the cysteine residues were conserved in the BRS virus G protein, a thirteen amino acid region reported
30 previously to be exactly conserved in the G proteins of subgroups A and B HRS viruses was not conserved in the BRS virus G protein. Only six of the thirteen amino acids in this region of the BRS virus G protein were conserved, and two of these six were the cysteine residues (Figure 3).

The amino acid identity among the BRS virus G protein and the HRS virus proteins was significantly lower than the amino acid identity observed between the G proteins of the HRS virus subgroup A and B (Figure 4). The overall amino acid identity between the HRS virus A2 and 18357 G proteins is 53%. The BRS virus G protein shared only 29% amino acid identity with the HRS virus A2 G protein and 30% amino acid identity with the HRS virus 18537 G protein. Comparison of amino acid identity within each of the three postulated domains of the G proteins showed distinct differences in the levels of identity in the three domains. The identity between the proposed extracellular domain of the BRS virus and HRS virus G proteins was significantly lower than the overall amino acid identity, and lower than the identity between extracellular domains of the two HRS virus G proteins (Figure 4). The proposed cytoplasmic and transmembrane domains of the BRS virus G protein were more conserved than the extracellular domain, with 43% and 55% amino acid identity, respectively, observed among the corresponding domains of either HRS virus G protein (Figure 4).

Although the overall identity of the BRS virus G protein to either HRS G protein was lower than that between HRS virus G proteins, there were similarities in the hydropathy profiles of the different G proteins (Figure 5) (Johnson et al., 1987, Proc. Natl. Acad. Sci. U.S.A. 84:5625-5629; Satake, et al., 1985, Nucleic Acids Res. 13:7795-7812; Wertz et al., 1985, Proc. Natl. Acad. Sci. U.S.A. 82:4075-4079). There was an initial hydrophilic region followed by a hydrophobic peak in the G protein of HRS virus A2, HRS virus 18537 and the G protein of BRS virus. These two regions together corresponded to the proposed cytoplasmic domain (amino acids 1-37). This was followed by a region of strong hydrophobicity, corresponding to the proposed transmembrane domain (amino

acids 38-66). The remainder of the protein was mainly hydrophilic, corresponding to the proposed extracellular domain (amino acids 67-257, 292, or 298). This hydrophilic extracellular domain was interrupted in all three G proteins by a short region of hydrophobicity (amino acids 166-188) which corresponded to the area containing the four conserved cysteine residues.

The BRS virus G mRNA contained two open reading frames in addition to the major open reading frame. One open reading frame began at nucleotide 212, ended at nucleotide 352, and coded for a predicted polypeptide of 46 amino acids. The other and larger of the two was in the same coding frame as the first but began at nucleotide 380 and ended at nucleotide 814. This open reading frame coded for a predicted polypeptide of 144 amino acids.

6.2.3. CONSTRUCTION AND CHARACTERIZATION OF RECOMBINANT VACCINIA VIRUS VECTORS CONTAINING THE BRS VIRUS G GENE

A cDNA containing the complete major open reading frame of the BRS virus G mRNA was inserted into a plasmid, pIB176-192, designed for the construction of vaccinia virus recombinants. The plasmid pIB176-192 is similar to recombination plasmids described previously (Ball, et al., 1986, Proc. Natl. Acad. Sci. USA 83:246-250) which contain a portion of the HindIII J fragment of vaccinia virus with the 7.5K promoter inserted into the thymidine kinase (tk) gene. However, in the case of pIB176-192, the 1710 base pair fragment between the HindIII and PvuII sites of the HindIII J fragment was inserted between the HindIII and SmaI sites of pIB176 and the 7.5K promoter directs transcription in the direction opposite of the tk promoter. The cDNA of the BRS virus G mRNA was inserted downstream of the major transcriptional start site of the 7.5K promoter. The HindIII J fragment containing the inserted BRS virus G

gene was inserted into the genome of vaccinia virus (Copenhagen strain) by homologous recombination (Stott, et al., 1986, J. Virol. 60:607-613). Thymidine kinase negative recombinant vaccinia viruses (rVV) were identified by hybridization of recombinant viral DNA with a probe
5 specific for the BRS virus G gene and selected by three rounds of plaque purification. Recombinant vaccinia virus G642 and G4567 contained the BRS virus G gene in the forward and reverse orientation with respect to the 7.5K promoter, respectively. The genome structures of
10 recombinant vaccinia viruses were confirmed by southern blot analysis of digests of vaccinia virus core DNA to confirm that the BRS virus G gene was inserted within the tk gene of the recombinant viruses.

15

6.2.4. ANALYSIS OF PROTEINS FROM CELLS
INFECTED WITH RECOMBINANT VACCINIA VIRUS
CONTAINING THE BRS VIRUS G GENE

The ability of the recombinant vaccinia virus containing the BRS virus G gene to express the BRS virus G
20 protein was examined in BT cells. The proteins from BT cells infected with either BRS virus, wild type vaccinia virus, or the recombinant vaccinia viruses containing the BRS virus G gene were analyzed by western blot analysis with BRS virus 391-2 specific antiserum because the BRS
25 virus G protein is not readily labeled with [³⁵S]-methionine due to the scarcity of this amino acid in the BRS virus G amino acid sequence, and the fact that the 391-2 antiserum does not work for immunoprecipitation. The 391-2 antiserum was shown previously to recognize the BRS
30 virus G protein in a western blot analysis of proteins from BRS virus infected cells (Lerch et al., 1989, J. Virol. 63:833-840). The serum recognized two proteins present in rVV G642 (forward orientation) infected cells (Figure 6, lane G642+) but not in rVV G4567 (reverse orientation) or

35

wild type vaccinia infected cells (Figure 6, lanes G4567- and VV respectively). The two proteins produced in rVV G642 infected cells comigrated with proteins recognized by the antiserum in BRS virus infected cells. One of the proteins in rVV G642 infected cells comigrated with the mature BRS virus G protein, migrating as a broad band between the 68kD and 97kD protein markers. The other protein migrated at approximately 43kD.

10 6.2.5. SURFACE EXPRESSION OF BRS VIRUS G
PROTEIN EXPRESSED FROM RECOMBINANT
VACCINIA VIRUSES

The G protein is a glycoprotein expressed on the surface of infected cells and incorporated in the membranes of virions (Huang, 1985, Virus Res. 2:157-173). In order to determine if the BRS virus G protein expressed in the recombinant vaccinia virus infected cells was transported to and expressed on the surface of infected cells, indirect immunofluorescent staining was performed on recombinant virus infected cells. BT cells were found to be extremely sensitive to vaccinia virus infection. There was high background fluorescence and very few cells survived the staining procedure. For these reasons immunofluorescence staining was done on recombinant virus infected HEP-2 cells. HEP-2 cells that were infected with recombinant G642 (Figure 7, panel rVVG) demonstrated specific surface fluorescence which was not present in either uninfected cells (Figure 7, panel M) or wild type vaccinia virus infected cells (Figure 7, panel VV).

30 6.3. DISCUSSION

The G protein of respiratory syncytial virus is an unusual viral glycoprotein for a variety of reasons. Although it has been characterized as the attachment protein for HRS virus (Levine et al., 1987, J. Gen. Virol. 68:2521-2524),

it lacks both neuraminidase and hemagglutinating activities observed in the attachment proteins of other viruses in the Paramyxovirus family (Gruber and Levine, 1983, J. Gen. Virol. 64:825-832; Richman et al., 1971, Appl. Microbiol. 21: 1099). Evidence suggests the HRS virus G protein is extensively glycosylated with approximately 55% of the mass of the mature protein estimated to be due to addition of O-linked oligosaccharide side chains (Lambert, 1988, Virology 164:458-466). The G protein of BRS virus has been shown to be antigenically distinct from the HRS virus G protein (Lerch et al., 1989, J. Virol. 63:833-840; Orvell et al., 1987, J. Gen. Virol. 68:3125-3135). In addition, with the exception of one report (Matumoto et al., Arch. Gesamte Virusforsch 44:280-290), BRS virus is unable to productively infect cells of human origin while HRS virus infects both human and bovine cells. It is possible that the difference in host range between BRS and HRS viruses may be reflected in the amino acid sequence differences observed in the attachment proteins.

In order to further examine the differences between the BRS virus and the HRS virus G proteins the nucleotide sequence of the BRS virus G protein mRNA from cDNA clones was determined. The BRS virus G mRNA was smaller than the G mRNAs of the HRS viruses, and shared 51% sequence identity with the G mRNAs of the HRS viruses sequenced to date. The consensus viral gene start and end sequences observed in HRS virus genes were conserved in the BRS virus G mRNA, as was the position of the initiation codon with respect to the initiation codon of the HRS virus G mRNA. The BRS virus G mRNA had a larger 3' noncoding region, which combined with the smaller size of the BRS virus G mRNA, resulted in a major open reading frame which coded for a polypeptide of 257 amino acids and having an estimated molecular weight of 28kDa. This compares to polypeptides of 298 and 292 amino acids coded for by the G

mRNAs of HRS virus subgroup A and subgroup B viruses, respectively, and estimated molecular weights of about 32kDa for each. The size of the predicted BRS virus G polypeptide when compared to the estimated size of the mature BRS virus G protein found in infected cells suggested that there is extensive modification of the BRS virus G polypeptide.

Studies using endoglycosidases, inhibitors of carbohydrate addition, and a cell line deficient in O-linked glycosylation have shown that the HRS virus G protein is extensively glycosylated with both N- and O-linked carbohydrate side chains (Lambert, 1988, *Virology* 164:458-466). The mature BRS virus G protein from infected cells was shown to be glycosylated and had an electrophoretic mobility similar to the 90kDa HRS virus G protein whereas the predicted amino acid sequence for the polypeptide indicated a protein of 28kDa (Lerch et al., 1989, *J. Virol.* 63:833-840; Westenbrink et al., 1989, *J. Gen. Virol.* 70:591-601). This suggested the BRS virus G protein was also extensively glycosylated as is the HRS virus G protein. The predicted amino acid sequence of the BRS virus G protein had high levels of serine and threonine (25%), similar to the levels for the HRS virus G proteins (30% and 28% for subgroup A and B, respectively) although the actual number of potential O-glycosylation sites for BRS virus (66) is lower than the 91 potential sites found in HRS virus subgroup A (Johnson et al., 1987, *Proc. Natl. Acad. Sci. U.S.A.* 84:5625-5629; Satake, et al., 1985, *Nucleic Acids Res.* 13:7795-7812; Wertz et al., 1985, *Proc. Natl. Acad. Sci. U.S.A.* 82:4075-4079). The high content of serine and threonine in the deduced BRS virus G amino acid sequence suggests that the BRS virus G protein also has the potential for extensive O-linked glycosylation.

Although the overall amino acid composition of the BRS virus G protein was similar to that of the HRS virus G protein, the BRS virus G amino acid sequence had a lower level of overall amino acid identity with the HRS virus G proteins of either the subgroup A or B viruses (Johnson et al., 1987, Proc. Natl. Acad. Sci. U.S.A. 84:5625-5629). There was only 29-30% identity between the BRS virus G protein and the G protein of either subgroup A or B HRS viruses, whereas there is 53% amino acid identity when the G proteins of the HRS virus subgroup A and subgroup B viruses are compared (Johnson et al., 1987, Proc. Natl. Acad. Sci. U.S.A. 84:5625-5629). Higher levels of amino acid identity were present between the BRS virus G protein and the HRS virus G proteins in the proposed cytoplasmic and transmembrane domains, but again this level was not as high as that found when comparing those regions of the G proteins of subgroup A and B HRS viruses (Figure 4). The fact that the BRS virus G protein amino acid sequence is not significantly more closely related to the G protein amino acid sequence of either HRS virus subgroup A or B suggests that the human and bovine respiratory syncytial viruses diverged prior to the emergence of the HRS virus A and B subgroups and that they should be classified in separate Pneumovirus subgroups.

The predicted amino acid sequence of the BRS virus G protein showed that the BRS virus and HRS virus G proteins shared only 29-30% amino acid identity. In spite of these differences, the hydropathy profiles of the two proteins showed strong similarities suggesting the possibility of similar overall structural features.

Johnson et al. (1987, Proc. Natl. Acad. Sci. U.S.A. 84:5625-5629) suggested that a conserved 13 amino acid region found in the extracellular domain of the G protein of HRS viruses could be a candidate for a receptor binding site. The fact that this conserved region is not conserved

in the G protein of BRS virus could relate to the host specificity of BRS virus. The four conserved cysteine residues found in the G protein of both the BRS virus and HRS viruses could result in a similar secondary structure among the G proteins with specific differences in the conserved region changing the host specificity. Convalescent calf serum has suggested the possibility that BRS virus may have antigenic subgroups as is HRS virus (Lerch et al., 1989, J. Virol. 63:833-840).

Recombinant vaccinia viruses containing a cDNA insert to the BRS virus G gene expressed the BRS virus G protein. This BRS virus G protein had an electrophoretic mobility in SDS-polyacrylamide gels which was similar to the G protein from BRS virus infected cells. Antiserum specific for BRS virus 391-2 recognized the BRS virus G protein produced by recombinant vaccinia virus in infected cells as shown by western blot analysis. The BRS virus G protein expressed from the recombinant vaccinia virus was transported to and expressed on the surface of infected cells as shown by surface immunofluorescence.

7. EXAMPLE: NUCLEOTIDE SEQUENCE ANALYSIS OF BOVINE RESPIRATORY SYNCYTIAL VIRUS FUSION PROTEIN mRNA AND A RECOMBINANT VACCINIA VIRUS

7.1. MATERIALS AND METHODS

7.1.1. VIRUS AND CELLS

The growth and propagation of BRS virus 391-2, wild type (Copenhagen strain) and recombinant vaccinia viruses, bovine nasal turbinate (BT) cells, HEp-2 cells, and thymidine kinase negative (tk⁻) 143B cells were as described in Hraby and Ball (1981, J. Virol. 40:456-464; Stott et al., 1986, J. Virol. 60: 607-613; Lerch et al., 1989, J. Virol. 63:833-840).

7.1.2. PROTEIN LABELING AND HARVEST OF BRS VIRUS INFECTED CELLS

Cell monolayers of bovine nasal turbinate (BT) cells were mock, BRS virus or HRS virus-infected. Tunicamycin (1.5µg/ml, Boehringer Mannheim) was added to the medium
5 covering the cells at 25 hours post infection where indicated. After one hour the medium was removed from all monolayers and replaced with DMEM lacking methionine (Gibco). Tunicamycin was re-added where indicated. Following a 30 minute incubation, [³⁵S]methionine
10 (100µCi/ml, New England Nuclear) was added to the medium. The cells were incubated for two hours and proteins harvested as described in Lerch et al., 1989, J. Virol. 63:833-840. Virus-specific proteins were immunoprecipitated as described in Wertz et al., (1985,
15 Proc. Natl. Acad. Sci. U.S.A. 82:4075-4079) using Wellcome anti-RS serum (Wellcome Reagents Ltd.). Proteins were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970, Nature (London) 227:680-685), and detected by fluorography (Davis and Wertz, 1982, J. Virol.
20 41:821-832).

7.1.3. CDNA SYNTHESIS, MOLECULAR CLONING, AND IDENTIFICATION OF F SPECIFIC cDNA CLONES

cDNAs were synthesized using the strand replacement
25 method of Gubler and Hoffman (1983, Gene 25:263-269) as described in D'Alessio et al. (1987, Focus 9:1-4). T4 DNA polymerase (BRL) was used to make the ends of the cDNAs blunt (Maniatis et al., 1982, in "Molecular Cloning: a laboratory manual," Cold Spring Harbor Laboratory, Cold
30 Spring Harbor, N.Y.). The cDNAs were ligated into M13mp19 replicative form (RF) DNA, which had been digested with SmaI and treated with calf intestinal alkaline phosphatase (Maniatis et al., supra), and transfected into competent E. coli DH5 αF' cells (Bethesda Research Laboratories)

(Hanahan, 1983, J. Mol. Biol. 166:557-580). M13mp19 phage containing BRS virus F specific inserts were identified by dot blot hybridization of phage DNA (Davis et al., 1986, "Basic Methods in Molecular Biology. Elsevier Science Publishing Co., Inc., New York, N.Y.) probed with a
5 previously identified BRS virus F gene specific clone (Lerch et al., 1989, J. Virol. 63:833-840) which had been labeled by nick translation (Rigby et al., 1977, J. Mol. Biol. 113:237-251). Growth and manipulations of M13mp19 and recombinant phage were as described by Messing (1983,
10 Meth. Enzymol. 101:20-78).

7.1.4. NUCLEOTIDE SEQUENCING AND PRIMER EXTENSION ON RNA

Dideoxynucleotide sequencing using the Klenow fragment
15 of E. coli DNA polymerase I (Pharmacia) or a modified T7 DNA Polymerase (Sequenase, U.S. Biochemicals) was done as described in Tabor and Richardson (1987, Proc. Natl. Acad. Sci. U.S.A. 84:4746-4771) and Lim and Pene (1988, Gene Anal. Tech. 5:32-39) using an M13 sequencing primer (New
20 England Biolabs). Extension of a synthetic DNA primer, complementary to bases 267 to 284 of the BRS virus F mRNA, was done on BRS virus mRNA using Avian myeloblastosis virus (AMV) reverse transcriptase (Molecular Genetic Resources) (Air, 1979, Virology 97:468-472). BRS virus mRNA used a
25 template in the primer extension on RNA was harvested as described for mRNA used for cDNA synthesis (Lerch et al., 1989, J. Virol. 63:833-840). Nucleotide sequencing and primer extension were done using [α -³⁵S] dATP (Amersham) and polyacrylamide-urea gradient gel electrophoresis as
30 described by Biggin et al. (1983, Proc. Natl. Acad. Sci. U.S.A. 80:3963-3965). The nucleotide sequence was analyzed using the University of Wisconsin Genetics Computer Group software package (Devereux et al., 1984, Nucl. Acids Res. 12:387-395).
35

-53-

7.1.5. SYNTHESIS AND CLONING OF A COMPLETE cDNA TO THE BRS VIRUS F mRNA

A cDNA containing the complete major open reading frame of the BRS virus F mRNA was synthesized using a specific synthetic oligonucleotide for second strand
5 synthesis. The nucleotide sequence for this oligonucleotide was determined from sequence analysis of BRS virus mRNAs. First strand synthesis occurred as described in D'Alessio et al. (1987, Focus 9:1-4). Following synthesis, the RNA template was digested with
10 RNase A (1µg/µl) for thirty minutes at 37°C. The resulting single-stranded cDNAs were isolated by phenol extraction and ethanol precipitation. The oligonucleotide used for second strand synthesis had the sequence
5' CACGGATCCACAAGTATGTCCAACC³' with the 5' first nine bases
15 of the oligonucleotide containing a BamHI restriction enzyme site. The single-stranded cDNAs were mixed with the oligonucleotide (50µg/ml), heated to 100°C for one minute and placed on ice. AMV reverse transcriptase was used to synthesize the second strand of the cDNAs in a reaction
20 [50mM Tris-HCl (pH8.0), 50mM KCl, 5mMgCl₂, 10mM dithiothreitol, 1.6mM dNTPs, 100U AMV reverse transcriptase] that was incubated for one hour at 50°C. The cDNAs were separated from protein by phenol extraction, recovered by ethanol precipitation, and the ends made blunt
25 with T4 DNA polymerase. Blunt-ended cDNAs were then digested with the restriction enzyme BamHI (Bethesda Research Laboratories) and separated from protein by phenol extraction. M13mp18 RF DNA, that was digested with BamHI and SmaI and treated with calf intestinal phosphatase, was
30 added to the cDNAs and recovered by ethanol precipitation. The cDNAs and vector were ligated and transfected into competent DH5αF' cells (Bethesda Research Laboratories) as described in Hanahan (1983, J. Mol. Biol. 166:557-580).

7.1.6. CONSTRUCTION AND ISOLATION OF RECOMBINANT VACCINIA VIRUS VECTORS

A complete cDNA clone, F20, corresponding to the BRS virus F mRNA was subcloned into a recombination plasmid, pIBI76-192, by digestion with BamHI and KpnI, treatment
5 with T4 DNA polymerase to make the ends of the cDNA blunt, and ligation into the unique SmaI site of pIBI76-192. The plasmid pIBI76-192 is similar to recombination plasmids described in Ball et al. (1986, Proc. Natl. Acad. Sci. U.S.A. 83:246-250). In the case of pIBI76-192, base pairs
10 (bp) 1 to 1710 of the HindIII J fragment of vaccinia virus was inserted between the HindIII and SmaI sites of pIBI76 (International Biotechnology Inc.). A 280 bp. fragment of DNA that contains the 7.5k promoter of vaccinia virus was inserted into the EcoRI site (bp 670 of the HindIII J
15 fragment) of the vaccinia virus thymidine kinase (tk) gene (bp 502 to 1070 of the HindIII J fragment, Weir and Moss, 1983, J. Virol. 46:530-537). The orientation of this promoter was such that it directed transcription from right to left on the conventional vaccinia virus map, opposite to
20 the direction of transcription of the vaccinia virus tk gene. The unique SmaI site in pIBI76-192 was downstream of the major transcriptional start site of the 7.5K promoter.

The isolation of recombinant vaccinia viruses containing the BRS virus F mRNA sequence was as described
25 in Stott et al. (1986, J. Virol. 60:607-613) except that the recombinant viruses were identified using the blot procedure of Lavi and Ektin (1981, Carcinogenesis 2:417-423).

30 7.1.7. CHARACTERIZATION OF RECOMBINANT VACCINIA VIRUS VECTORS

DNA from vaccinia virus cores isolated from wild type and recombinant viruses was prepared for southern blot analysis as described by Esposito et al. (1981, J. Virol.

-55-

Meth. 2:175-179). Restriction enzyme digestion, southern blot analysis, and radioactive labeling of DNA by nick translation were as described in Section 6, supra. Metabolic labeling of proteins from wild type and recombinant vaccinia virus infected cells was as above
5 except that infected cells were exposed to label for three hours starting at three hours postinfection. Proteins were analyzed by SDS-polyacrylamide gel electrophoresis using standard procedures. Immunoprecipitation of virus-specific proteins was as described in Wertz et al. (1985, Proc.
10 Natl. Acad. Sci. U.S.A. 82:4075-4079) using Wellcome anti-RS serum (Wellcome Reagents Ltd.). For immunofluorescence HEp-2 cells were grown on glass cover slips. Cells were infected with wild type or recombinant vaccinia viruses (moi=10) and at 24 hours postinfection the cells were
15 stained by indirect immunofluorescence using anti-BRS virus 391-2 serum (Lerch et al., 1989, J. Virol. 63:833-840) as a first antibody followed by fluorescein conjugated anti-bovine IgG (H+L). Fluorescence was observed through a
20 Nikon fluorescence microscope.

7.2. RESULTS

7.2.1. NUCLEIC ACID SEQUENCE AND COMPARISON

In order to determine the complete nucleotide sequence
25 of the BRS virus F mRNA, cDNA clones in M13 phage vectors were isolated and their nucleotide sequence analyzed. The BRS virus F mRNA sequence was determined from eight clones derived independently from four separate cDNA synthesis reactions. The areas of the BRS virus F mRNA sequence
30 determined from the different clones are shown in Figure 8. The majority of the sequence was determined from three clones, FB3, FB5 and F20. Clone F20 is a full length BRS virus F cDNA which was synthesized using oligonucleotide specific for the 5' end of the BRS virus F mRNA. The
35 inserts from clones FB3, FB5 and F20 were excised using the

restriction enzymes PstI and KpnI, which do not cut within the inserts, and subcloned into M13mp18 RF DNA to sequence from the opposite end of the cDNA. In addition, restriction fragments of the inserts were subcloned into M13mp18 and mp19 RF DNA to allow for determination of the sequence of the middle of the cDNAs. Clone F20 was digested with the restriction enzymes AlwNI, PflMI, EcoRV, or HpaI, and clones FB3 and FB5 were digested with the restriction enzyme EcoRI. The 5' end of the BRS virus F mRNA sequence was determined by extension of a DNA oligonucleotide on mRNA from BRS virus infected cells. The sequence for the oligonucleotide, complementary to bases 267 to 284 of the BRS virus F mRNA, was determined from the sequence provided by the cDNA clones. Clones F29, FB2, F138 and FB1 were all 750 nucleotides or less were not sequenced extensively.

The BRS virus F mRNA contained 1899 nucleotides excluding a polyadenylate tail (Figure 9). Seven bases at the exact 5' end of the mRNA could not be determined due to strong stop signals in all four nucleotide reactions for each base during primer extension on mRNA. The sequence at the 3' end of the BRS virus F mRNA conformed to one of the two consensus gene-end sequences, 5'AGU-AU-UpolyA3',
A A
U U

found at the end of all HRS virus genes (Collins et al., 1986, Proc. Natl. Acad. Sci. U.S.A. 83:4594-4598) and at the 3' end of the BRS virus G mRNA. The BRS virus F mRNA had a single major open reading frame starting with an initiation codon beginning at nucleotide 14 and extending to a termination codon at nucleotide 1736. There was a 161 nucleotide noncoding region at the 3' end prior to the 3' polyadenylate tract (Figure 9).

The nucleotide sequence of the BRS virus F mRNA was compared to the published sequences for the F mRNA of HRS virus A2 (Collins et al., 1986, Proc. Natl. Acad. Sci.

U.S.A. 83:4594-4598), Long (Lopez et al., 1988, Virus Res. 10:249-262), RSS-2 (Baybutt and Pringle, 1987, J. Gen. Vir. 68:2789-2796) and 18537 (Johnson and Collins, 1988, J. Gen. Virol. 69:2623-2628) to determine the extent of nucleic acid identity among the different F mRNA sequences (Table 1).

5

10

15

20

25

30

35

TABLE 1
NUCLEIC ACID IDENTITY BETWEEN THE FUSION
PROTEIN GENES OF RESPIRATORY SYNCYTIAL VIRUSES

VIRUSES COMPARED	(% IDENTITY WITHIN INDICATED AREAS)		
	TOTAL	CODING SEQUENCE	3' NONCODING SEQUENCE
HRS VIRUS, SUBGROUP A vs HRS VIRUS, SUBGROUP A	97-98	97	98-99
HRS VIRUS, SUBGROUP B vs HRS VIRUS, SUBGROUP A	79	82	47
BRS VIRUS vs HRS VIRUS, SUBGROUP A	72	75	36-37
BRS VIRUS vs HRS VIRUS, SUBGROUP B	71	74	39

HRS virus A2, Long and RSS-2 are subgroup A viruses, and 18537 is a subgroup B virus (Anderson et al., 1985, J. Infect. Dis. 151:626-633; Mufson et al., 1985, J. Gen. Virol. 66:2111-2124). The level of nucleic acid identity between the F mRNAs of BRS virus and HRS viruses (71.5%) was
5 similar to that observed when comparing the F mRNAs of the two HRS virus subgroups (79%). Both the level of identity between the F mRNAs of BRS virus and HRS virus, and between the F mRNAs of the two HRS virus subgroups was lower than the level of identity between the F mRNAs of HRS viruses
10 within the same subgroups (97-98%). The level of nucleotide sequence identity between the BRS virus and HRS viruses in the 3' noncoding region of the F sequence was 37.5% compared to 74.5% in the F sequence coding region. This was similar to the levels of identity in 3' noncoding and coding
15 regions, 47% and 82% respectively, when comparing the F sequences of the subgroup A HRS viruses to the subgroup B HRS virus. There was variation in the nucleotides at two positions in the F mRNA sequence. In one clone, clone F20, nucleotides 455 and 473 were a G and C, respectively, rather
20 than the A and G observed in the other clone and shown in the sequence (Figure 9).

7.2.2. PREDICTED AMINO ACID SEQUENCE OF THE BRS
VIRUS F PROTEIN AND COMPARISON TO THE F
PROTEINS SEQUENCES OF HRS VIRUS

25

The open reading frame of the BRS virus F mRNA predicted a polypeptide of 574 amino acids. The amino acid sequence of this polypeptide is shown below the mRNA sequence in Figure 9. The estimated molecular weight of the
30 predicted BRS virus F polypeptide was 63.8kDA. A hydropathy profile of the predicted polypeptide indicated strong hydrophobic regions at the amino terminus, corresponding to residues 1 through 26, and close to the carboxy terminus, corresponding to residues 522 through 549 (Figure 10). The

35

hydropathy profile and amino acid sequence suggested domains in the BRS virus F protein similar to those described for the HRS virus F protein, including an amino terminal signal peptide region (residues 1-26), carboxy terminal anchor region (residues 522-549), and putative cleavage sequence⁵ (residues 131-136) that generates the F₁ and F₂ polypeptides (see Figure 10 and 11). There were three potential sites for addition of N-linked carbohydrate side chains, two in the proposed F₂ polypeptide, and one in the proposed F₁ polypeptide (Figure 9).

¹⁰ The predicted BRS virus F amino acid sequence was compared to the predicted amino acid sequences of the F polypeptides of HRS viruses A2 (Collins et al., 1984, Proc. Natl. Acad. Sci., U.S.A. 81:7i683-7i687), Long (Lopez et al., 1988, Virus Res. 10:249-262), RSS-2 (Baybutt and¹⁵ Pringle, 1987, J. Gen. Virol. 68:2789-2796) and 18537 (Johnson and Collins, 1988, J. Gen. Virol. 69:2623-2628) (Figure 11). The BRS virus F polypeptide was 574 amino acids as are all four HRS virus F polypeptides. Proposed carboxy terminal hydrophobic anchor (residues 522-549) and²⁰ amino terminal signal regions (residues 1-26) present in the BRS virus F protein were similar to those in the HRS virus F proteins. In addition, the sequence of Lys-Lys-Arg-Lys-Arg-Arg at residues 131 to 136 in the BRS virus F protein represented a proposed cleavage signal. This sequence was²⁵ identical to the proposed cleavage signals in all the HRS virus F proteins. The cleavage sequence in the BRS virus F protein was followed by a stretch of hydrophobic residues (residues 137-158) that would represent the amino terminus of the F₁ polypeptide following cleavage. The nucleotide³⁰ differences in clone F20 would result in amino acids residues 148 and 154, part of the proposed F₁ amino terminus, changing from Val to Ile, and Leu to Val, respectively. These differences would result in the amino

acids at these two positions in the BRS virus F protein being identical to the corresponding amino acid positions in the HRS virus F proteins.

With the exception of one cysteine residue (residue 25) in the proposed amino terminal signal peptide, all the⁵ cysteine residues were conserved in position among the BRS virus and HRS virus F proteins. This includes the cysteine residue at position 550, which has been shown to be the site of covalent attachment of palmitate to the human RS virus F protein (Arumugham et al., 1989, J. Biol. Chem. 264: 10339-¹⁰10342). There was a single potential site for N-linked glycosylation (residue 500) in the F₁ polypeptide of the BRS virus F protein that was conserved in all the HRS virus F proteins (Figure 11). There were two potential sites for N-linked glycosylation (residues 27 and 120) in the BRS¹⁵ virus F₂ polypeptide (Figure 9 and 11). The potential site at residue 27 was conserved among the BRS virus and HRS virus F proteins (Figure 11). However, the HRS virus F₂ polypeptides contained a total of four or five potential sites, depending on the isolate, and the position of the²⁰ remaining potential site for N-linked glycosylation in the BRS virus F₂ polypeptide was not conserved in all the HRS virus F₂ polypeptides (Figure 11). The existence of only two potential N-linked glycosylation sites on the BRS virus F₂ polypeptide was consistent with the earlier observation²⁵ that there were differences in electrophoretic mobility of the BRS virus and HRS virus F₂ polypeptides (Lerch et al., 1989, J. Virol. 63:833-840).

The amino acid identity between the F proteins of the different viruses is shown for the different regions of the³⁰ F protein (Table 2).

TABLE 2
AMINO ACID IDENTITY BETWEEN THE FUSION PROTEINS OF RESPIRATORY SYNCYTIAL VIRUSES
(% IDENTITY WITHIN INDICATED AREAS)

VIRUSES COMPARED	TOTAL	F ₁ PEPTIDE	F ₂ PEPTIDE	SIGNAL PEPTIDE
HRS VIRUS, SUBGROUP A vs HRS VIRUS, SUBGROUP A	97-98	98-99	93-96	81-88
HRS VIRUS, SUBGROUP B vs HRS VIRUS, SUBGROUP A	89	93	83	36
BRS VIRUS vs HRS VIRUS, SUBGROUP A	80	88	67-68	4
BRS VIRUS vs HRS VIRUS, SUBGROUP B	81	88	68	12

HRS viruses A2, Long, and RSS-2 are subgroup A viruses, and 18537 is a subgroup B virus (Anderson et al., 1985, J. Infect. Dis. 151:626-633); Mufson et al., 1985, J. Gen. Virol. 66:2111-2124). Although the greatest extent of variation was in the proposed signal peptide region⁵ (residues 1-26), the overall hydrophobicity of this region was conserved in the BRS virus F protein (Figure 10). The proposed F₂ polypeptide of the BRS virus F protein showed a lower level of identity to the F₂ polypeptides than is present between the F₂ polypeptides of the HRS virus A and B¹⁰ subgroups (Johnson and Collins, 1988, J. Gen. Virol. 2623-2628). In contrast, the levels of identity between the different F₁ polypeptides were similar whether comparing BRS virus to HRS virus or comparing two HRS virus subgroups.

¹⁵ 7.2.3. EFFECTS OF TUNICAMYCIN ON ELECTROPHORETIC MOBILITY OF BRS VIRUS F PROTEIN

To determine whether previously observed glycosylation of the BRS virus F protein (Lerch et al., 1989, J. Virol. 63:833-840) was due to N-linked glycosylation, the²⁰ electrophoretic mobility of the BRS virus F protein radioactively labeled in the presence and absence of tunicamycin, an inhibitor of N-linked glycosylation, was examined. Proteins in BRS virus, HRS virus, and mock infected cells were radioactively labeled by exposure to²⁵ [³⁵S]methionine in the presence and absence of tunicamycin, immunoprecipitated and separated by SDS-polyacrylamide gel electrophoresis. BRS virus F protein labeled in the presence of tunicamycin demonstrated a change in electrophoretic mobility compared to BRS virus F protein³⁰ labeled in the absence of tunicamycin (Figure 12, lanes B_T and B). In addition, the BRS virus F₀, F₁ and F₂ polypeptides synthesized in the presence of tunicamycin had electrophoretic mobilities similar to the respective HRS virus F₀, F₁ and F₂ polypeptides synthesized in the presence³⁵

of tunicamycin (Figure 12, lanes H_T and B_T). These results indicated that the BRS virus F protein was glycosylated via N-linked carbohydrate additions, and the observed differences in the electrophoretic mobility of the BRS virus and HRS virus F_2 polypeptides are due to differences in the extent of glycosylation as predicted by the deduced amino acid sequence (Figure 11).

7.2.4. CONSTRUCTION AND ISOLATION OF RECOMBINANT VACCINIA VIRUS VECTORS CONTAINING THE BRS VIRUS GENE

10 To facilitate the study of the role of individual proteins of BRS virus in eliciting a protective immune response in the host, the BRS virus F gene was placed in a vaccinia virus expression vector. A cDNA (F20) containing the complete major open reading frame of the BRS virus F
15 mRNA was inserted into a plasmid, pIBI76-192, designed for construction of vaccinia virus recombinants. The plasmid pIBI76-192 is similar to recombination plasmids described in Ball et al. (1986, Proc. Natl. Acad. Sci. U.S.A. 83:246-250) that contain a portion of the HindIII J fragment of vaccinia
20 virus with the 7.5K promoter inserted into the thymidine kinase (tk) gene. However, in the case of pIBI76-192, the 7.5K promoter directs transcription in the opposite direction of transcription of the tk gene. The cDNA of the BRS virus F mRNA was inserted downstream of the major
25 transcriptional start site of the 7.5K promoter. The HindIII J fragment containing the inserted BRS virus F gene was inserted into the genome of vaccinia virus (Copenhagen strain) by homologous recombination (Stott et al., 1986, J. Virol. 60:607-613). Thymidine kinase negative recombinant
30 vaccinia viruses (rVV) were identified by hybridization of recombinant viruses with a probe specific for the BRS virus F gene and selected by three rounds of plaque purification. Recombinant vaccinia virus F464 and F1597 contained the BRS virus F gene in the forward and reverse orientation with

respect to the 7.5K promoter, respectively. The genome structures of recombinant vaccinia viruses were examined by Southern blot analysis of restriction enzyme digests of vaccinia virus core DNA. These experiments confirmed that the BRS virus F gene was inserted within the tk gene of the recombinant viruses.

7.2.5. ANALYSIS OF PROTEINS FROM CELLS INFECTED
WITH RECOMBINANT VACCINIA VIRUS CONTAINING
THE BRS VIRUS F GENE

The ability of the recombinant vaccinia viruses containing the BRS virus F gene to express the BRS virus F protein was examined in tissue culture cells. BT cells were infected with either BRS virus, wild type vaccinia virus, or the recombinant vaccinia viruses containing the BRS virus F gene in the positive or negative orientation with respect to the promoter. The proteins in cells were labeled by incorporation of [³⁵S]methionine, harvested, and then immunoprecipitated with the Wellcome anti-RS serum and separated by SDS-polyacrylamide gel electrophoresis. The recombinant vaccinia virus F464 (forward orientation) produced at least two proteins in infected cells which were precipitated by the Wellcome anti-RS serum (Figure 13, lane F464+), and were not present in wild type vaccinia virus infected cells (Figure 13, lane VV) or rVV F1597 (reverse orientation) infected cells (Figure 13, lane F1597-). The two proteins specific to rVVF464 infected cells had electrophoretic mobilities identical to the BRS virus F₀ and F₁ polypeptides. A cellular protein that was precipitated by the serum (Figure 12, lane M), had an electrophoretic mobility similar to the BRS virus F₂ polypeptide and inhibited the detection of an F₂ polypeptide in rVV F464 infected cells. It was presumed that if the F₀ protein was

produced and cleaved to generate the F_1 polypeptide, the F_2 polypeptide was also present even though it could not be visualized.

An additional protein that had been previously observed in BRS virus infected cells (Lerch et al., 1989, J. Virol. 53:833-840) and was slightly larger than the BRS virus 22K protein was observed in rVV F464 infected cells (Figure 13, lane F464+). This additional protein was only produced in BRS virus infected cells or rVV F464 infected cells and was immunoprecipitated by the Wellcome antiserum. This result 10 indicated that the additional protein may be either specific cleavage fragment of the BRS virus F protein or interact with the BRS virus F protein.

7.2.6. GLYCOSYLATION OF THE BRS VIRUS F PROTEIN EXPRESSED 15 FROM A RECOMBINANT VACCINIA VIRUS

In order to determine whether the F polypeptides synthesized in the recombinant virus infected cells were glycosylated in a manner similar to the authentic BRS virus F polypeptides, the proteins in rVV F464 infected BT cells 20 were labeled with [35 S]methionine in the presence and absence of tunicamycin. These proteins were compared to similarly labeled proteins from BRS virus infected BT cells by immunoprecipitation and SDS-polyacrylamide gel electrophoresis (Figure 14). In the presence of 25 tunicamycin, the F_0 and F_1 polypeptides produced in rVV F464 virus infected cells had faster electrophoretic mobilities than their counterparts synthesized in the absence of tunicamycin (Figure 14, compare lane F_T to lane F), and had electrophoretic mobilities identical to the unglycosylated 30 F_0 and F_1 polypeptides from BRS virus infected cells (Figure 13, lane F_T compared to lane B_T). In the presence of tunicamycin, the protein band from recombinant virus infected cells which presumably contained the BRS virus F_2 polypeptide along with a cellular protein disappeared, and 35

there was an increase in intensity of a band at the bottom of the gel (Figure 14, lane F_T) where the unglycosylated BRS virus F₂ migrates (see Figure 12).

5 7.2.7. CELL SURFACE EXPRESSION OF THE BRS
 VIRUS F PROTEIN EXPRESSED FROM
 RECOMBINANT VACCINIA VIRUS

 The HRS virus F glycoprotein is expressed on the surface of infected cells and incorporated in the membranes of virions (Huang, 1983, "The genome and gene products of human respiratory syncytial virus" Univ. of North Carolina at Chapel Hill; Huang et al., 1985, 2:157-173). In order to determine if the BRS virus F protein expressed in the recombinant vaccinia virus infected cells was transported to and expressed on the surface of infected cells, recombinant vaccinia virus infected cells were examined by indirect immunofluorescence staining. BT cells were extremely sensitive to vaccinia virus infection and could not be used for immunofluorescence without high background fluorescence. For this reason immunofluorescence was carried out on recombinant virus infected HEp-2 cells. The antiserum used in the immunofluorescence was BRS virus 391-2 specific antiserum which was shown previously to recognize the BRS virus F protein in Western blot analysis of proteins from BRS virus infected cells (Lerch et al., 1989, J. Virol. 63:833-840). This antisera was specific in immunofluorescence assays for BRS virus infected, but not uninfected cells. HEp-2 cells that were infected with recombinant F-464 (Figure 15, panel rVVF) demonstrated specific surface fluorescence that was not present in either uninfected cells (Figure 15, panel M) or wild type vaccinia virus infected cells (Figure 15, panel VV).

7.3. DISCUSSION

We have determined the nucleotide sequence of cDNA clones corresponding to the BRS virus F mRNA. The nucleotide sequence and deduced amino acid sequence were compared to that of the corresponding HRS virus sequences.

⁵The F mRNA was identical in length, 1899 nucleotides, to the HRS virus A2, Long and RSS-2 F mRNAs (Collins et al., 1984, Proc. Natl Acad. Sci. U.S.A. 81:7683-7687; Baybutt and Pringle, 1987, J. Gen. Virol. 68:2789-2796; Lopez et al. 1988, Virus Res. 10: 249-262). The HRS virus 18537 F mRNA

¹⁰is three nucleotides shorter in the 3' noncoding region (Johnson and Collins, 1988, J. Gen. Virol. 69:2623-2628). The level of nucleic acid identity between the BRS virus and HRS virus F mRNAs was similar to the level between the F mRNAs of the HRS virus subgroup A and B viruses (Johnson and

¹⁵Collins, 1988, J. Gen. Virol. 69:2623-2628). The major open reading frame of the BRS virus F mRNA encoded a predicted protein of 574 amino acids, identical in size to the HRS virus F proteins (Collins et al., 1984, Proc. Natl Acad. Sci. U.S.A. 81:7683-7687; Baybutt and Pringle, 1987, J.

²⁰Gen. Virol. 68:2789-2796; Johnson and Collins, 1988, J. Gen. Virol. 69:2623-2628; Lopez et al., 1988, Virus Res. 10:249-262). The predicted major structural features of the BRS virus F protein, such as an N-terminal signal, a C-terminal anchor sequence, and a cleavage sequence to yield F₁ and F₂

²⁵polypeptides were conserved with these features in the HRS virus F protein. The deduced amino acid sequence of the BRS virus F protein had 80% overall amino acid identity to the HRS virus F proteins. The BRS virus and HRS virus F₁

³⁰polypeptides were more conserved, with 88% amino acid identity, than the F₂ polypeptides which had 68% amino acid identity. If BRS virus and HRS virus have diverged from a single common ancestor, the lower levels of amino acid identity in F₂ compared to F₁ suggest there may be

³⁵different, or fewer constraints on the F₂ polypeptide to

maintain a specific amino acid sequence than on the F₁ polypeptide. Also, the difference in the levels of identity among the human and bovine F₂ polypeptides in comparison to the F₁ polypeptides suggests that conservation of the exact amino acid sequence of the F₂ polypeptide is not as
5 important as that of the F₁ polypeptide amino acid sequence in maintaining the structure and function of the F protein. The amino acid sequence of the proposed anchor region and amino terminus of the F₁ polypeptide of BRS virus were highly conserved when compared to those sequences in the HRS
10 virus F proteins. The proposed amino terminal signal peptides of the BRS virus and HRS virus F proteins were not conserved in amino acid sequence but were conserved in predicted hydrophobicity.

Synthesis of the BRS virus F polypeptides in the
15 presence of tunicamycin, an inhibitor of N-linked glycosylation, demonstrated that the BRS virus F polypeptides were glycosylated by the addition of N-linked carbohydrate moieties. Also, the F₂ polypeptides of BRS virus and HRS virus synthesized in the presence of
20 tunicamycin had the same electrophoretic mobility in SDS-polyacrylamide gels. This indicated the BRS virus and HRS virus F₂ polypeptides had differences in the extent of glycosylation. Nucleotide sequence analysis of cDNA clones to the BRS virus F mRNA confirmed a difference in the number
25 of potential glycosylation sites. The deduced BRS virus F₂ amino acid sequence contained only two sites for potential N-linked oligosaccharide addition, whereas there are four potential sites in the F₂ polypeptide of HRS virus A2.

The use of tunicamycin to inhibit N-linked
30 glycosylation showed that the difference in the electrophoretic mobility of HRS virus and BRS virus F₁ polypeptides was not due to glycosylation differences as there were slight differences in the electrophoretic
35 mobilities of the unglycosylated F₁ polypeptides of BRS

virus and HRS virus. Nucleotide sequence analysis showed the predicted BRS virus F₁ amino acid sequence and HRS virus F₁ polypeptides were of the same size and both contained one potential site for N-linked oligosaccharide addition. At present it is concluded that slight differences which exist in the amino acid compositions of the BRS virus and HRS virus F₁ polypeptides caused the difference in electrophoretic migration. In support of this conclusion is recent work that demonstrates that changing a single amino acid in the vesicular stomatitis virus G protein, while not changing the glycosylation of the protein alters its electrophoretic mobility (Pitta et al., 1989, J. Virol. 63:3801-3809).

The BRS virus F protein, and the HRS virus F proteins have conserved epitopes. Both convalescent calf serum and monoclonal antibodies will recognize the F protein from either virus (Orvell et al., 1987, J. Gen. Virol. 68:3125-3135; Stott et al., 1984, Dev. Biol. Stand. 57:237-244; Kennedy et al., 1988, J. Gen. Virol. 69:3023-3032; Lerch et al., 1989, J. Virol. 63:833-840). Orvell et al. (1987, J. Gen. Virol. 68:3125-3135) found that only 3 out of 35 monoclonal antibodies generated to an HRS virus F protein did not recognize the F protein of three BRS virus strains. In addition, all of the 11 monoclonal antibodies against the F protein which were neutralizing for HRS virus also neutralized the infectivity of the BRS virus strains (Orvell et al., 1987, J. Gen. Virol. 68:3125-3135). Studies using synthetic peptides and monoclonal antibodies have suggested that at least two epitopes on the HRS virus F protein are involved in neutralization of the virus. The epitopes are positioned at amino acids 212 to 232 (Trudel et al., 1987a, J. Gen. Virol. 68:2273-2280; Trudel et al., 1987b, Canad. J. Microbiol. 33:933-938) and amino acids 283 to 299. The first of these epitopes is exactly conserved in the BRS virus F protein. In the second epitope, there are three

changes in the BRS virus F protein, all of which are conservative changes. Although both of these epitopes are on the F₁ polypeptide, amino acids affecting neutralization have been localized to the F₂ polypeptide in the fusion protein of Newcastle disease virus (Totoda et al., 1988, J. Virol. 62:4427-4430; Neyt et al., 1989, J. Virol. 63:952-954).

In contrast to the similarities of the HRS virus and BRS virus F proteins, the G proteins of these viruses are antigenically distinct. All monoclonal antibodies generated
10 against the G protein of either HRS virus subgroup did not recognize the BRS virus G protein (Orvell et al., 1987, J. Gen. Virol. 68:3125-3135). It has been shown that polyclonal convalescent serum from a calf infected with BRS virus, while recognizing the BRS virus G protein, did not
15 recognize the G protein of a HRS virus (Lerch et al., 1989, J. Virol. 63:833-840). The antigenic similarity between the BRS virus and HRS virus F proteins and the difference in antigenic cross reactivity between the BRS virus and HRS virus G proteins was also reflected in the levels of amino
20 acid identity between the homologous proteins of HRS virus and BRS virus. The HRS virus and BRS virus G proteins shared only 30% amino acid identity whereas the F proteins of the two viruses shared 80% amino acid identity. The differences in the F and G glycoproteins are also evident in
25 their presentation of epitopes. Garcia-Barreno et al. (1989, J. Virol. 63:925-932), using panels of monoclonal antibodies, found that the epitopes of the F protein divided into five nonoverlapping groups, whereas the competition profiles of many of the epitopes on the G protein are
30 extensively overlapped.

Recombinant vaccinia viruses containing a cDNA insert to the BRS virus F gene expressed the BRS virus F protein. This BRS virus F protein was cleaved into F₁ and F₂
35 polypeptides, and had an electrophoretic mobility in SDS-

polyacrylamide gels which was similar to the F protein from BRS virus infected cells. Experiments with tunicamycin, an inhibitor of N-linked glycosylation, demonstrated that the BRS virus F protein expressed from recombinant infected cells was glycosylated to a level similar to the F proteins⁵ from BRS virus infected cells. The BRS virus F protein expressed from the recombinant vaccinia virus was transported to and expressed on the surface of infected cells as shown by surface immunofluorescence.

10 8. EXAMPLE: PRODUCTION OF MONOSPECIFIC, POLYCLONAL
 ANTIBODY TO THE BRS VIRUS G PROTEIN AND
 DEMONSTRATION OF ANTIGENIC SPECIFICITY

 To test the biological activity of the bovine RS virus attachment protein expressed from the recombinant VV vectors and to assess the antigenic cross-reactivity between the BRS¹⁵ and HRS virus G proteins using a polyclonal antisera, recombinant VV expressing either the BRS virus or HRS virus G protein were used to immunize animals as described in Stott et al., 1986, J. Virol. 60:607-613. Sera from animals immunized with the BRS virus G protein specifically²⁰ immunoprecipitated the BRS virus attachment protein, but did not recognize the human RSG protein (Figure 16). Similarly, antisera raised against the HRS virus G protein was specific for the HRS virus G protein and showed no recognition of the²⁵ bovine RS virus G protein, confirming the antigenic distinctness of the two attachment proteins.

30

35

9. DEPOSIT OF MICROORGANISMS

The following [microorganisms] were deposited with the American Type Culture Collection, Rockville, Maryland.

plasmid pRLG414-76-191

5 plasmid pRLF2012-76-1902

plasmid pRLNB3-76

virus rVG-642

virus rVF-464

The present invention is not limited in scope by the
10 microorganisms deposited or the embodiments disclosed in the
examples which are intended as illustrations of a few
aspects of the invention and any embodiments which are
functionally equivalent are within the scope of this
invention. Indeed, various modifications of the invention
15 in addition to those shown and described herein will become
apparent to those skilled in the art and are intended to
fall within the scope of the appended claims. A number of
publications have been cited herein, which are incorporated
by reference in their entirety.

20

25

30

35

International Application No: PCT/

/

MICROORGANISMSOptional Sheet in connection with the microorganism referred to on page 73, line _____ of the description ***A. IDENTIFICATION OF DEPOSIT ***Further deposits are identified on an additional sheet ☒ *

Name of depository institution *

American Type Culture Collection

Address of depository institution (including postal code and country) *

12301 Parklawn Drive
Rockville, MD 20852

Date of deposit *

July 10, 1990

Accession Number *

ATCC 40841

B. ADDITIONAL INDICATIONS * (leave blank if not applicable). This information is continued on a separate attached sheet ☐**C. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE *** (If the indications are not for all designated States)**D. SEPARATE FURNISHING OF INDICATIONS *** (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later * (Specify the general nature of the indications e.g., "Accession Number of Deposit")

E. ☐ This sheet was received with the International application when filed (to be checked by the receiving Office)*Mamie Arson*
(Authorized Officer)☐ The date of receipt (from the applicant) by the International Bureau *

(Authorized Officer)

American Type Culture Collection
12301 Parklawn Drive
Rockville, MD 20852

Date of Deposit: July 10, 1990

ATCC 40842

Date of Deposit: July 10, 1990

ATCC 40843

Date of Deposit: July 10, 1990

ATCC VR 2276

Date of Depssit: July 10, 1990

ATCC VR 2277

WHAT IS CLAIMED IS:

1. A recombinant DNA molecule comprising a nucleic acid sequence encoding bovine respiratory syncytial virus G protein.
5
2. The recombinant DNA molecule of claim 1 in which the bovine respiratory syncytial virus G protein encoding nucleic acid sequence or subsequence is substantially as depicted in Figure 2 and comprises at least about ten
10 nucleotides.
3. The recombinant DNA molecule of claim 1 in which the bovine respiratory syncytial virus G protein encoding nucleic acid sequence or subsequence is substantially as
15 depicted in Figure 2 from about nucleotide 16 to about nucleotide 912 and comprises at least about ten nucleotides.
4. The recombinant DNA molecule of claim 2 as
20 contained in plasmid pRLG414-76-191 deposited with the ATCC and with the accession number 40841.
5. A recombinant DNA molecule comprising a sequence encoding a protein substantially homologous to the amino
25 acid sequence depicted in Figure 3, or portions thereof comprising at least about ten nucleotides.
6. The recombinant DNA molecule of claim 1, 2, or 5 in which the expression of nucleic acid sequence encoding
30 bovine respiratory syncytial virus G protein or peptide fragment is regulated by a second nucleic acid sequence so that bovine respiratory syncytial virus G protein is expressed in a host transformed with the recombinant DNA molecule.

-75-

7. The recombinant DNA molecule of claim 6 as contained in rVG642 deposited with the ATCC and having accession number VR 2276.

5 8. A recombinant microorganism containing the recombinant DNA molecule of claim 1, 2, or 5.

9. The recombinant microorganism of claim 8 which is a bacterium.

10 10. The recombinant microorganism of claim 8 which is a yeast.

11. A recombinant microorganism containing the
15 recombinant DNA molecule of claim 6.

12. A eukaryotic cell containing the recombinant DNA molecule of claim 1, 2, or 5.

13. A eukaryotic cell containing the recombinant DNA
20 molecule of claim 6.

14. A eukaryotic cell containing the recombinant DNA molecule of claim 7.

25 15. A method for producing bovine respiratory syncytial virus G protein or a fragment thereof comprising growing a recombinant microorganism containing the DNA molecule of claim 1, 3, or 5, such that the DNA molecule is
30 expressed by the microorganism, and isolating the expressed bovine respiratory syncytial virus G protein or fragment.

16. A method for producing bovine respiratory syncytial virus G protein or a fragment thereof comprising
35 growing a recombinant microorganism containing the DNA

-76-

molecule of claim 6, such that the DNA molecule is expressed by the microorganism, and isolating the expressed bovine respiratory syncytial virus G protein or fragment.

5 17. A method for producing bovine respiratory syncytial virus G protein or a fragment thereof comprising growing a recombinant microorganism containing the DNA molecule of claim 1, 3, or 5, such that the DNA molecule is expressed by the eukaryotic cell and isolating the expressed
10 bovine respiratory syncytial virus G protein or fragment.

18. A method for producing bovine respiratory syncytial virus G protein or a fragment thereof comprising growing a recombinant microorganism containing the DNA
15 molecule of claim 6, such that the DNA molecule is expressed by the eukaryotic cell and isolating the expressed bovine respiratory syncytial virus G protein or fragment.

19. A method for producing bovine respiratory
20 syncytial virus G protein or a fragment thereof comprising growing a recombinant microorganism containing the DNA molecule of claim 7, such that the DNA molecule is expressed by the eukaryotic cell and isolating the expressed bovine respiratory syncytial virus G protein or fragment.

25 20. The method according to claim 15 in which the microorganism is a bacterium.

21. The method according to claim 16 in which the
30 microorganism is a bacterium.

-77-

22. The product of claim 15.
23. The product of claim 16.
- 5 24. The product of claim 17.
25. The product of claim 18.
26. The product of claim 19.
- 10 27. A subunit vaccine comprising the product of claim
15 in a suitable pharmaceutical carrier.
28. A subunit vaccine comprising the product of claim
15¹⁶ in a suitable pharmaceutical carrier.
29. A subunit vaccine comprising the product of claim
17 in a suitable pharmaceutical carrier.
- 20 30. A subunit vaccine comprising the product of claim
18 in a suitable pharmaceutical carrier.
31. A subunit vaccine comprising the product of claim
19 in a suitable pharmaceutical carrier.
- 25 32. A subunit vaccine comprising the product of claim
20 in a suitable pharmaceutical carrier.
33. A recombinant virus which is infectious but
30 nonpathogenic and which comprises the DNA molecule of claim
1, 2, or 5.
34. A recombinant virus which is infectious but
nonpathogenic and which comprises the DNA molecule of claim
35.

-78-

35. A vaccine comprising the recombinant virus of claim 33.

36. A vaccine comprising the recombinant virus of claim 34.

37. A recombinant DNA molecule comprising a nucleic acid sequence encoding bovine respiratory syncytial virus F protein.

38. The recombinant DNA molecule of claim 37 in which the bovine respiratory syncytial virus F protein encoding nucleic acid sequence or subsequence is substantially as depicted in Figure 9 and comprises at least about ten nucleotides.

39. The recombinant DNA molecule of claim 37 in which the bovine respiratory syncytial virus F protein encoding nucleic acid sequence or subsequence is substantially as depicted in Figure 9 from about nucleotide 14 to about nucleotide 1735 and comprises at least about ten nucleotides.

40. The recombinant DNA molecule of claim 38 as contained in plasmid pRLF2012-76-1902 deposited with the ATCC and with the accession number 40842.

41. A recombinant DNA molecule comprising a sequence encoding a protein substantially homologous to the amino acid sequence depicted in Figure 11 or portions thereof comprising at least about ten nucleotides.

42. The recombinant DNA molecule of claim 37, 38, or 41 in which the expression of nucleic acid sequence encoding bovine respiratory syncytial virus F protein or peptide

-79-

fragment is regulated by a second nucleic acid sequence so that bovine respiratory syncytial virus F protein is expressed in a host transformed with the recombinant DNA molecule.

5

43. The recombinant DNA molecule of claim 42 as contained in rVF-464 deposited with the ATCC and having accession number VR 2277 .

10

44. A recombinant microorganism containing the recombinant DNA molecule of claim 37, 38, or 41.

45. The recombinant microorganism of claim 44 which is a bacterium.

15

46. The recombinant microorganism of claim 44 which is a yeast.

20

47. A recombinant microorganism containing the recombinant DNA molecule of claim 42.

48. A eukaryotic cell containing the recombinant DNA molecule of claim 37, 38, or 41.

25

49. A eukaryotic cell containing the recombinant DNA molecule of claim 42.

50. A eukaryotic cell containing the recombinant DNA molecule of claim 43.

30

51. A method for producing bovine respiratory syncytial virus F protein or a fragment thereof comprising growing a recombinant microorganism containing the DNA molecule of claim 37, 38, or 41, such that the DNA molecule

35

is expressed by the microorganism, and isolating the expressed bovine respiratory syncytial virus F protein or fragment.

5 52. A method for producing bovine respiratory syncytial virus F protein or a fragment thereof comprising growing a recombinant microorganism containing the DNA molecule of claim 42, such that the DNA molecule is expressed by the microorganism, and isolating the expressed
10 bovine respiratory syncytial virus F protein or fragment.

53. A method for producing bovine respiratory syncytial virus F protein or a fragment thereof comprising growing a recombinant microorganism containing the DNA
15 molecule of claim 37, 38, or 41, such that the DNA molecule is expressed by the eukaryotic cell and isolating the expressed bovine respiratory syncytial virus F protein or fragment.

20 54. A method for producing bovine respiratory syncytial virus F protein or a fragment thereof comprising growing a recombinant microorganism containing the DNA molecule of claim 42, such that the DNA molecule is expressed by the eukaryotic cell and isolating the expressed
25 bovine respiratory syncytial virus F protein or fragment.

55. A method for producing bovine respiratory syncytial virus F protein or a fragment thereof comprising growing a recombinant microorganism containing the DNA
30 molecule of claim 43, such that the DNA molecule is expressed by the eukaryotic cell and isolating the expressed bovine respiratory syncytial virus F protein or fragment.

56. The method according to claim 51 in which the
35 microorganism is a bacterium.

-81-

57. The method according to claim 52 in which the microorganism is a bacterium.

58. The product of claim 51.

5 59. The product of claim 52.

60. The product of claim 53.

10 61. The product of claim 54.

62. The product of claim 55.

63. A subunit vaccine comprising the product of claim
15 51 in a suitable pharmaceutical carrier.

64. A subunit vaccine comprising the product of claim
52 in a suitable pharmaceutical carrier.

20 65. A subunit vaccine comprising the product of claim
53 in a suitable pharmaceutical carrier.

66. A subunit vaccine comprising the product of claim
54 in a suitable pharmaceutical carrier.

25 67. A subunit vaccine comprising the product of claim
55 in a suitable pharmaceutical carrier.

68. A subunit vaccine comprising the product of claim
30 56 in a suitable pharmaceutical carrier.

69. A recombinant virus which is infectious but
nonpathogenic and which comprises the DNA molecule of claim
37, 38, or 41.

35

70. A recombinant virus which is infectious but nonpathogenic and which comprises the DNA molecule of claim 42.

5 71. A vaccine comprising the recombinant virus of claim 69.

72. A vaccine comprising the recombinant virus of claim 70.

10 73. A recombinant or synthetic protein having an amino acid sequence substantially as depicted in Figure 3 or a subsequence thereof comprising an antigenic determinant.

15 74. A recombinant or synthetic protein having an amino acid sequence substantially as depicted in Figure 11 or a subsequence thereof comprising an antigenic determinant.

20 75. A subunit vaccine comprising the recombinant or synthetic protein of claim 73 in a suitable pharmaceutical carrier.

25 76. A subunit vaccine comprising the recombinant or synthetic protein of claim 74 in a suitable pharmaceutical carrier.

77. An antibody produced by a method comprising inoculating an animal with the recombinant or synthetic protein of claim 73.

30 78. The antibody of claim 77 which is a monoclonal antibody.

35 79. An antibody fragment or antibody derivative produced from the antibody of claim 77.

-83-

80. An antibody fragment or antibody derivative produced from the antibody of claim 78.

81. An antibody produced by a method comprising inoculating an animal with the recombinant or synthetic protein of claim 74.

82. The antibody of claim 81 which is a monoclonal antibody.

83. An antibody fragment or antibody derivative produced from the antibody of claim 81.

84. An antibody fragment or antibody derivative produced from the antibody of claim 82.

85. A method of diagnosing bovine respiratory syncytial virus infection comprising detecting the presence of bovine respiratory syncytial virus nucleic acid in a sample obtained from an animal potentially infected by bovine respiratory syncytial virus.

86. The method according to claim 85 comprising:

- (i) exposing nucleic acid prepared from a sample obtained from an animal potentially infected by bovine respiratory syncytial virus to a nucleic acid molecule having a sequence or subsequence substantially as depicted in Figure 2 and comprising at least about ten nucleotides under conditions permissive for hybridization; and
- (2) detecting whether hybridization has occurred,

in which detection of hybridization is indicative of bovine respiratory syncytial virus infection.

87. The method according to claim 85 comprising:

5 (i) exposing nucleic acid prepared from a sample obtained from an animal potentially infected by bovine respiratory syncytial virus to a nucleic acid molecule having a sequence or subsequence substantially as depicted in Figure 9 and comprising at least about ten nucleotides under conditions permissive for hybridization; and

10 (2) detecting whether hybridization has occurred,

in which detection of hybridization is indicative of bovine respiratory syncytial virus infection.

15 88. The method according to claim 86 in which RNA is prepared from an animal potentially infected by bovine respiratory syncytial virus and subjected to Northern blot analysis in step (i).

20 89. The method according to claim 87 in which RNA is prepared from an animal potentially infected by bovine respiratory syncytial virus and subjected to Northern blot analysis in step (i).

25 90. A method of diagnosing bovine respiratory syncytial virus infection comprising:

30 (i) exposing a serum sample obtained from an animal potentially infected by bovine respiratory syncytial virus to a recombinant or synthetic protein having an amino acid sequence substantially as depicted in Figure 3 or a subsequence thereof comprising an antigenic determinant under conditions permissive for binding of antibody to
35 protein; and

-85-

(ii) detecting whether binding of antibody to the protein has occurred, in which binding of antibody to protein is indicative of exposure to or infection by bovine respiratory syncytial virus.

91. The method according to claim 90 which is an enzyme linked immunosorbent assay.

92. The method according to claim 90 which comprises detecting the presence of antibody using Western blot techniques.

93. A method of diagnosing bovine respiratory syncytial virus infection comprising:

(i) exposing a serum sample obtained from an animal potentially infected by bovine respiratory syncytial virus to a recombinant or synthetic protein having an amino acid sequence substantially as depicted in Figure 11 or a subsequence thereof comprising an antigenic determinant under conditions permissive for binding of antibody to protein; and

(ii) detecting whether binding of antibody to the protein has occurred, in which binding of antibody to protein is indicative of exposure to or infection by bovine respiratory syncytial virus.

94. The method according to claim 93 which is an enzyme linked immunosorbent assay.

-86-

95. The method according to claim 93 which comprises detecting the presence of antibody using Western blot techniques.

5 96. A method of inducing an immune response toward bovine respiratory syncytial virus comprising administering an effective amount of the subunit vaccine of claim 27 to an animal in need of such treatment.

10 97. A method of inducing an immune response toward bovine respiratory syncytial virus comprising administering an effective amount of the subunit vaccine of claim 28 to an animal in need of such treatment.

15 98. A method of inducing an immune response toward bovine respiratory syncytial virus comprising administering an effective amount of the subunit vaccine of claim 29 to an animal in need of such treatment.

20 99. A method of inducing an immune response toward bovine respiratory syncytial virus comprising administering an effective amount of the subunit vaccine of claim 30 to an animal in need of such treatment.

25 100. A method of inducing an immune response toward bovine respiratory syncytial virus comprising administering an effective amount of the subunit vaccine of claim 31 to an animal in need of such treatment.

30 101. A method of inducing an immune response toward bovine respiratory syncytial virus comprising administering an effective amount of the subunit vaccine of claim 32 to an animal in need of such treatment.

-87-

102. A method of inducing an immune response toward bovine respiratory syncytial virus comprising administering an effective amount of the vaccine of claim 35 to an animal in need of such treatment.

5

103. A method of inducing an immune response toward bovine respiratory syncytial virus comprising administering an effective amount of the vaccine of claim 36 to an animal in need of such treatment.

10

104. A method of inducing an immune response toward bovine respiratory syncytial virus comprising administering an effective amount of the subunit vaccine of claim 63 to an animal in need of such treatment.

15

105. A method of inducing an immune response toward bovine respiratory syncytial virus comprising administering an effective amount of the subunit vaccine of claim 64 to an animal in need of such treatment.

20

106. A method of inducing an immune response toward bovine respiratory syncytial virus comprising administering an effective amount of the subunit vaccine of claim 65 to an animal in need of such treatment.

25

107. A method of inducing an immune response toward bovine respiratory syncytial virus comprising administering an effective amount of the subunit vaccine of claim 66 to an animal in need of such treatment.

30

108. A method of inducing an immune response toward bovine respiratory syncytial virus comprising administering an effective amount of the subunit vaccine of claim 67 to an animal in need of such treatment.

35

-88-

109. A method of inducing an immune response toward bovine respiratory syncytial virus comprising administering an effective amount of the subunit vaccine of claim 68 to an animal in need of such treatment.

5

110. A method of inducing an immune response toward bovine respiratory syncytial virus comprising administering an effective amount of the vaccine of claim 71 to an animal in need of such treatment.

10

111. A method of inducing an immune response toward bovine respiratory syncytial virus comprising administering an effective amount of the vaccine of claim 72 to an animal in need of such treatment.

15

112. A recombinant DNA molecule comprising a nucleic acid sequence encoding bovine respiratory syncytial virus N protein.

20

113. The recombinant DNA molecule of claim 112 in which the bovine respiratory syncytial virus N protein encoding nucleic acid sequence or subsequence is substantially as depicted in Figure 17 and comprises at least about ten nucleotides.

25

114. The recombinant DNA molecule of claim 113 as contained in plasmid pRLNB3-76 deposited with the ATCC and with the accession number 40843.

30

115. A recombinant DNA molecule comprising a sequence encoding a protein substantially homologous to the amino acid sequence depicted in Figure 18 or portions thereof comprising at least about ten nucleotides.

35

116. A recombinant microorganism containing the recombinant DNA molecule of claim 113.

117. The recombinant microorganism of claim 116 which
5 is a bacterium.

118. The recombinant microorganism of claim 116 which is a yeast.

119. A eukaryotic cell containing the recombinant DNA
10 molecule of claim 113.

120. A recombinant virus which is infectious but nonpathogenic and which comprises the DNA molecule of claim
15 113.

121. A vaccine comprising the recombinant virus of claim 120.

122. A recombinant or synthetic protein having an amino acid sequence substantially as depicted in Figure 18 or a subsequence thereof comprising an antigenic
20 determinant.

123. A subunit vaccine comprising the recombinant or synthetic protein of claim 122 in a suitable pharmaceutical
25 carrier.

124. An antibody produced by a method comprising
30 inoculating an animal with the recombinant or synthetic protein of claim 122.

125. The antibody of claim 124 which is a monoclonal antibody.

-90-

126. An antibody fragment or antibody derivative produced from the antibody of claim 125.

127. The method according to claim 85 comprising:

- 5 (i) exposing nucleic acid prepared from a sample obtained from an animal potentially infected by bovine respiratory syncytial virus to a nucleic acid molecule having a sequence or subsequence substantially as depicted in Figure 17 and comprising at least about ten nucleotides under conditions permissive for hybridization; and
- 10 (2) detecting whether hybridization has occurred,
- 15 in which detection of hybridization is indicative of bovine respiratory syncytial virus infection.

128. A method of diagnosing bovine respiratory syncytial virus infection comprising:

- 20 (i) exposing a serum sample obtained from an animal potentially infected by bovine respiratory syncytial virus to a recombinant or synthetic protein having an amino acid sequence substantially as depicted in Figure 18 or a subsequence thereof comprising an antigenic determinant under conditions permissive for binding of antibody to protein; and
- 25 (ii) detecting whether binding of antibody to the protein has occurred,
- 30 in which binding of antibody to protein is indicative of exposure to or infection by bovine respiratory syncytial virus.

-91-

129. A method of inducing an immune response toward bovine respiratory syncytial virus comprising administering an effective amount of the subunit vaccine of claim 123 to an animal in need of such treatment.

5

10

15

20

25

30

35

1/26

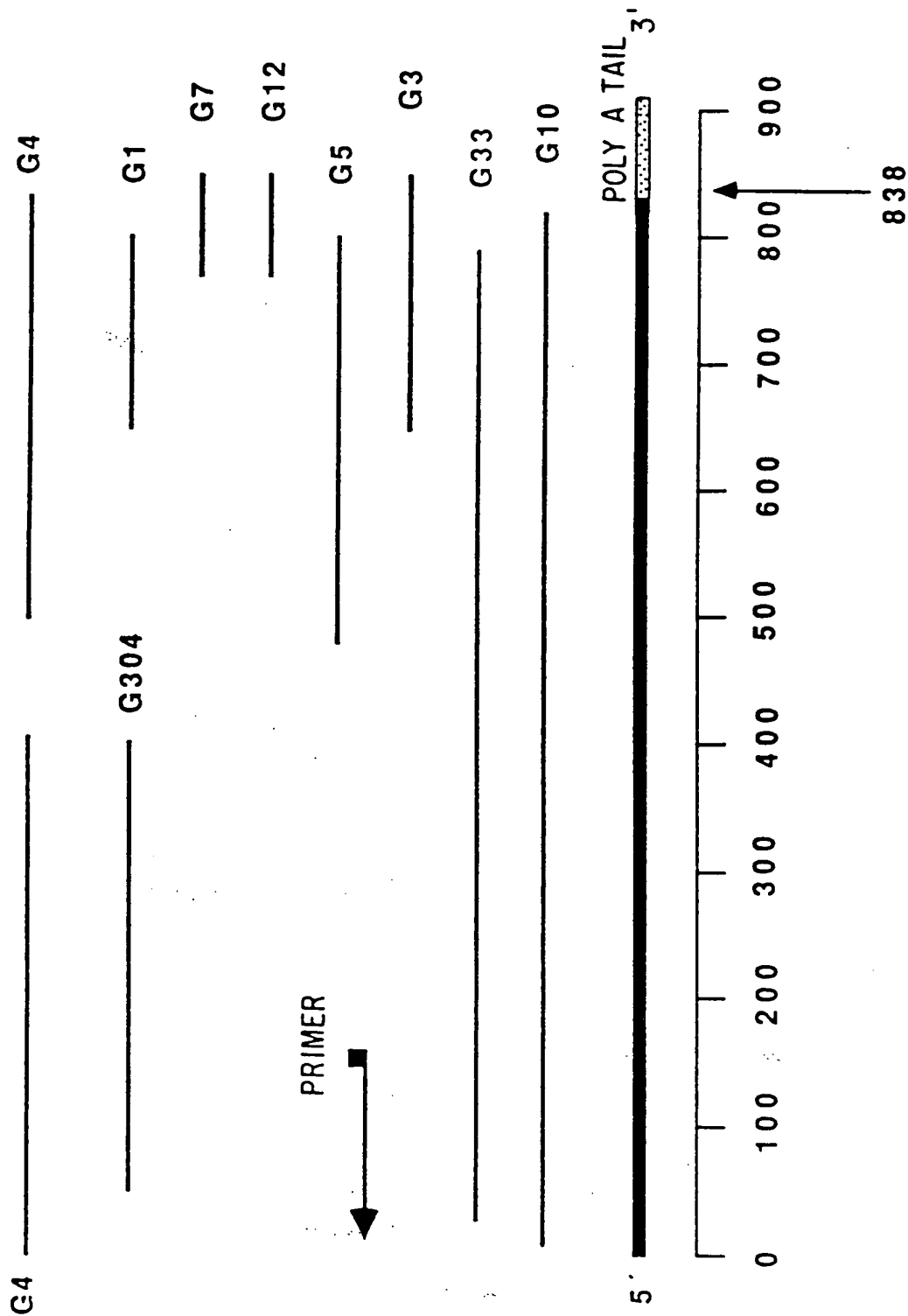


FIG. 1

2/26

A2 G G AC
 AA C AGG C A GC CCGCT G A A C G CA TCTC TC 80
 Bovine NCGGCAATACAAAGTATGTTCCAAACCATACCCCATCATCTTAAATTCAAGACATTAAAGAGGGCTTGGAAAGCCTCAAAATA 80
 18537 NNNNN G CC A C AGA A GC CTGC G TC TG A A A C G TA TCTT TC 80

A2 T AT T CAT C G C A C T TG A CA TCA A A TT G 160
 Bovine CTTTATAGTAGGATTATCATGTTTATATAAGTTCAATTTAAATCCCTTGTCCAAACGGCTTTGTCCACCCCTAGCAATGA 160
 18537 TC A T AT CC T C GA A TA A CA TA AC AGTT G 160

A2 T CA T TA A TG C A T AGCCTC C CCAC T CA A A TGC ATC 240
 Bovine TAACCTTGACATCACTCGTCATCACAGCCATTATTACATTAGTGTGGAAATGCTAAAGCCCAAGCCACATCCAAACCA 240
 18537 T CA C T A A TG A A T C TCTCT CC CAC TT CA TA A GGTTA 240

A2 TACAAG TGC AGC AT A G ACA C A ACC CC A TCCTC GCTTGG TCAGTC C 320
 Bovine ACCATCCAACAACACAAACAGCCCAAAACCATACCTCACCATTTTTCACAGAGCACAACATACTCAACTCACACATC 320
 18537 GTTCAAAC AT A A C CA TG AA C T CA C ACC T TC AGT CCACCAG AGGTCA T 320

A2 T A CGTCTGAA TTACA A A CACC C CTAG T AC AC CAGGA AGTC A CCT C 396
 Bovine AATTCAAAGCACCACACTGTCCCAACTACTAAACATAGACACTACTAGAGGAATTACATATGCTCACTCAACCAACGAAA 400
 18537 C AA CC A CACA A C A C AC CA ATTCAG C AT C CA ATACA AATC GA AC C 396

FIG. 2A

3/26

A2 T C TC GA C AAA CAAC A A A ACAAC C G CA C CA A A CGCCA A A 476
 Bovine CCCAAACAGAAAAAATCAAAGGCCA....ATCCACTCTACCCGCCACCAAAACCAATCAATCCATCGGGAAGCAT 476
 18537 AT C C C A C A G GAATCA TC A ACAG AC G AGC CA AT CGTTC AA A 476

A2 A A AAGC A CCA TA TG TTTC TG GTGT AA T A C C T CAGCAAC CAA 556
 Bovine CCCCCCTGAAAACCATCAAGACCACACAACACTTCCAAACACTCCCTATGTGCCTTGCAGTACATGTGAAGGTAATCTTG 556
 18537 T A AA A CAA TG TT C T TG GTGT AAT T T C T T GTAA AAC 556

A2 C GGG TA A A GAATAC AA C A AA G A AGAAA C T C AG C C A AA CC 636
 Bovine CTTGCTTATCACTCTGCCATATTGAGACGGAGAGCACCAGCAGAGCCCTACAAATCACCCCTCAAAGAGCTCCAAA 636
 18537 TC AA CA A A CAATAC AAGC ACAA AG A AAA A C AA C C C AA CC 636

A2 T ...G A C AGATC A CTC A C C AA T A G AGTA ACC C A GC CA GAAG 713
 Bovine CCCAA...AACCACATAAAA...GCCAACCAAGACAAACAATCCACCACAGACCCTGAAACCAACTGCAACCTA 709
 18537 A AACC A AC GAGACC AAC CCAGC AATG A A AG AAT ATC C A CC A AAA 716

A2 GCC ACCAT A C A A ACATCAT A TA A A TCACCTC A CAC GGA C GA CT A 793
 Bovine AAAACAACACAGCAACTCCACAACAA...GGCATCCTCTCTTCAACAGAACATCACACAAATCAATCACTA..CACAG 783
 18537 CC ACC T AG C A AGA G GACACCAGCATT A AATCCACCGTGC GACAC C C AA TA A 796

FIG. 2B

4/26

A2 G A ATGA AC T CTC T CTCCG AG CAA CC AGCC C CAAGTCTCTACAACATCCGAGTACCCA 873
 Bovine ATC TACAACACACCTCCCATATAACATCTAATTATNGTTCTATATATAGTTATT 838
 18537 TC A CAGCA TC CTC CA CTCCG AAACA ACCCAGCTCCACACAAATACCCACAGCATCCGAGCCCTCC 876

A2 TCACAACCTTCATCTCCACCCCAACACACACCCAGCCAG TACTTACTT 918
 Bovine
 18537 ACATTAAATCCTAAT TAAAAAAACCTAGTCACATGCTTAGTTATTC 921

FIG. 2C

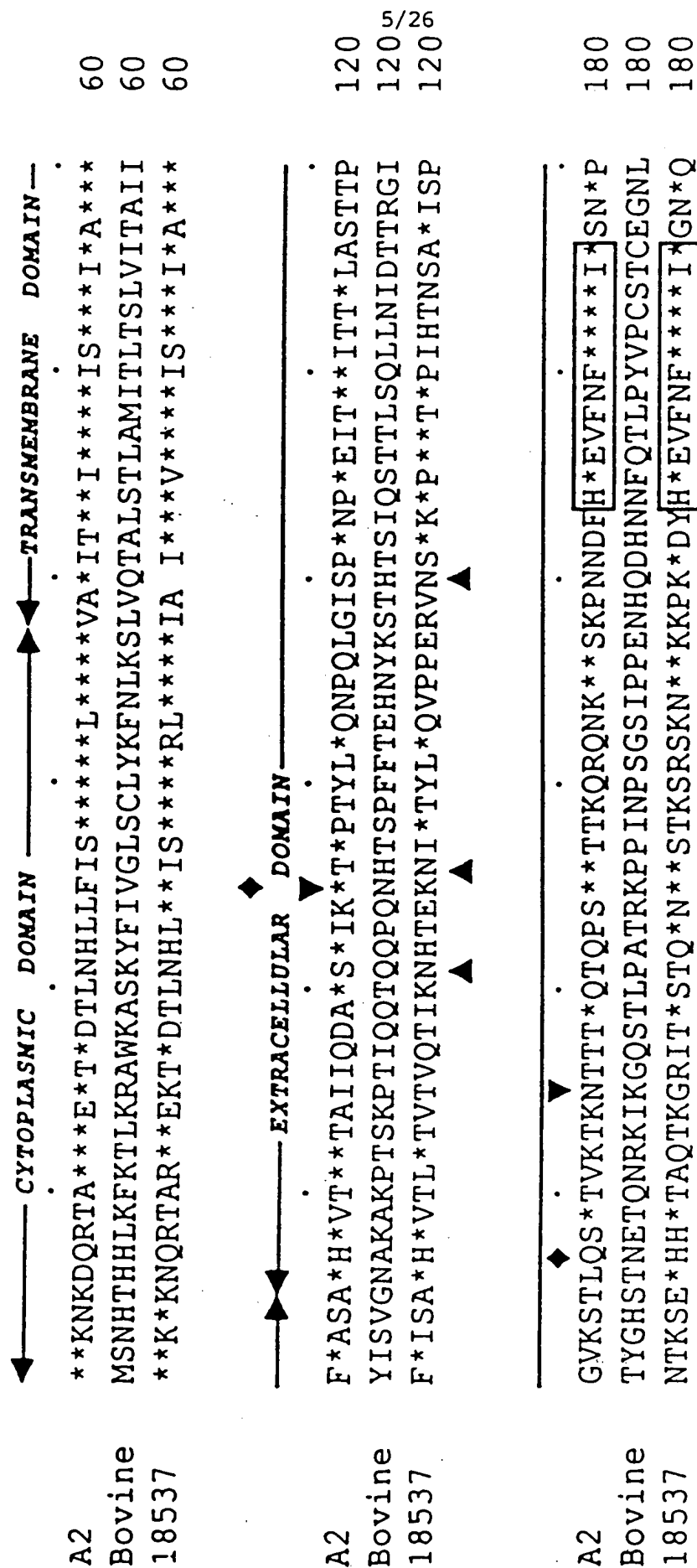


FIG. 3A

6/26

A2	T*WAI*KRIPNKK*GKKT*TKPT*K*TL***-**DP*PQTTKSKEVP*TKPTEEP*INTT	239
Bovine	ACLSLCHIEETERAPSRAPTITLKKTPKPKTT-KKPTKTTIHHRTSPETKLQPKNNATPQ	239
18537	L*K*I*KTIPSNK*KKK**KPTNK*TT***N*RDP**PAKMPKKEIITNPA*KP*LKTT	240
<hr/>		
A2	KTNIIT*LLTS*TTGNPELTSQMETFHSTSSSEGNPSPSQVSTTSEYPSQPSSPPNTPRQ	298
Bovine	QGILSSTEHTNQSTTQI	257
18537	ERDT*ISQSTVLDTI*PKYTIQQQLHSTSENTPSSSTQIPTASEPSTLNPN	292

FIG. 3B

	CYTO- PLASMIC TRANS- MEMBRANE		EXTRACELLULAR	COOH	TOTAL
	NH ₂				
<u>BOVINE VS</u> <u>HUMAN A2</u>	43%	55%	23%		29%
<u>BOVINE VS</u> <u>HUMAN 18537</u>	43%	55%	29%		34%
<u>HUMAN A2 VS</u> <u>HUMAN 18537</u>	81%	86%	44%		53%

7/26

FIG. 4

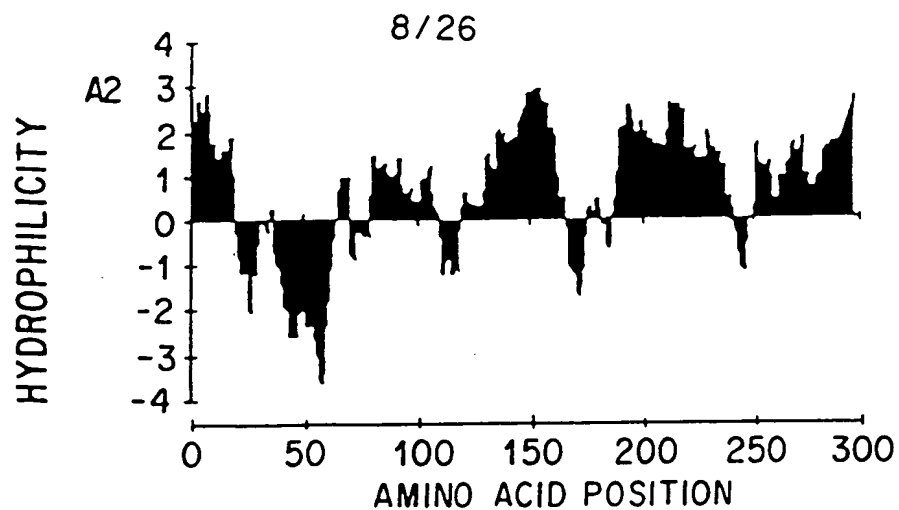


FIG. 5A

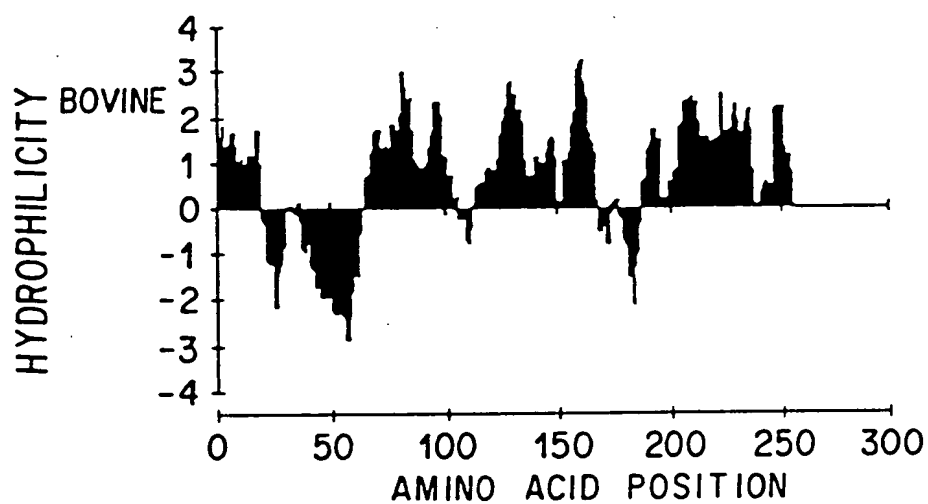


FIG. 5B

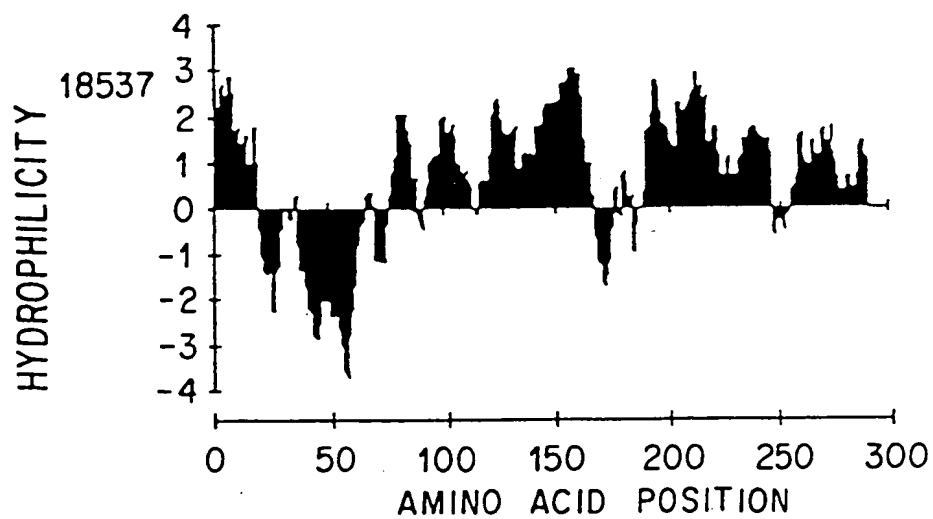


FIG. 5C

9/26

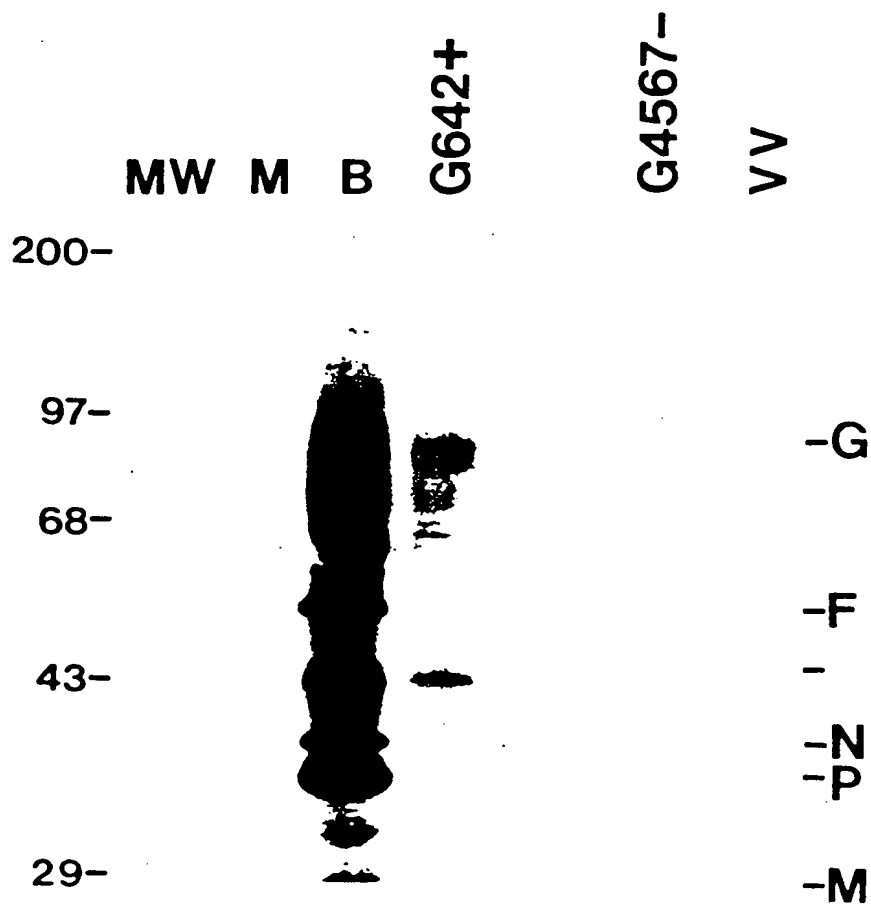
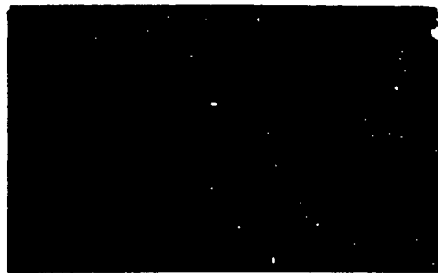


FIG. 6

10/26

M



VV



rVVG

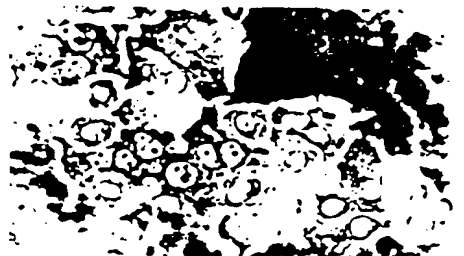


FIG. 7

11/26

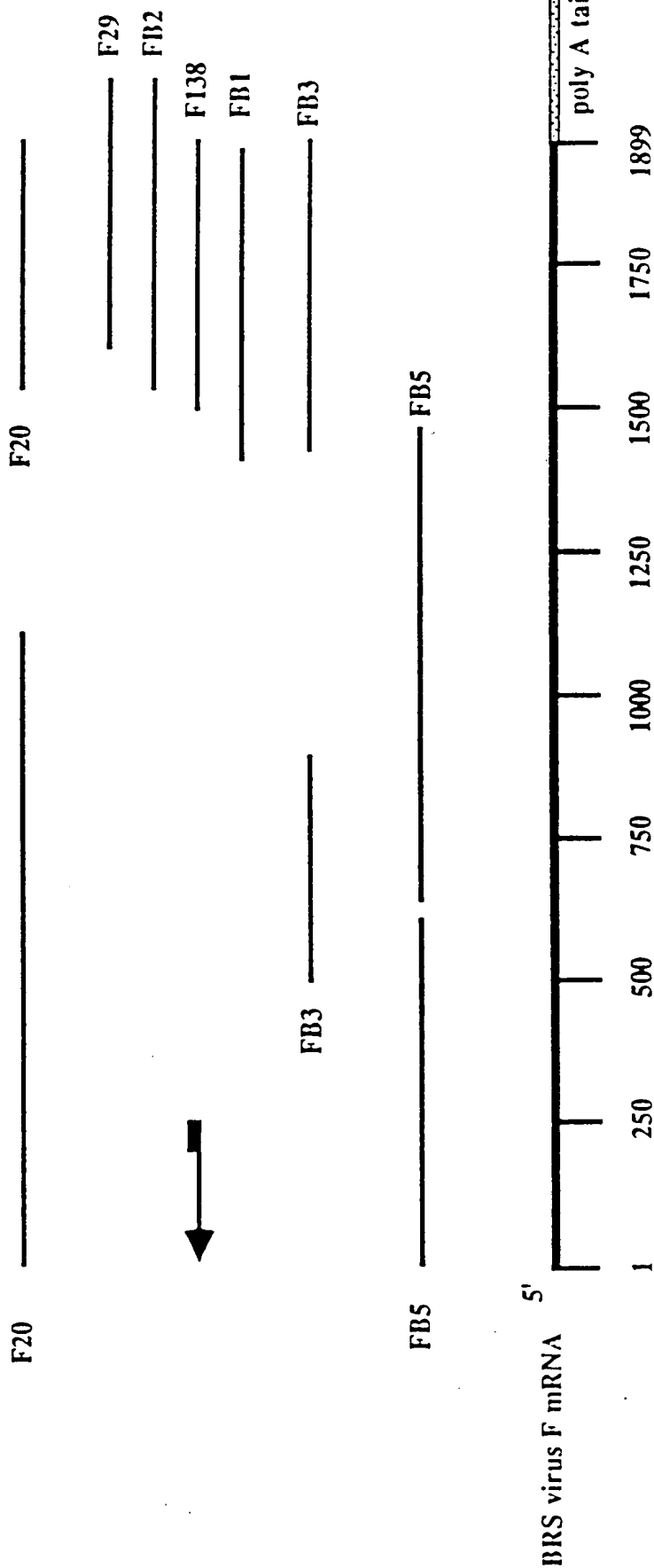


FIG. 8

NNNNNNATAAGGATGCGGCAACAGCCATGAGGATGATCATCAGCATTATCTTCTACCTATATGACACATATCACTTTA 85
MetAlaAlaThrAlaMetArgMetIleIleSerIleIlePheIleSerThrTyrMetThrHisIleThrLeu 24
 TGCCAAACATAACAGAAGAAATTTTATCAATCAACATGCAGTGCAGTGTAGAGGTTATCTTAGTGCATTAAAGAACTGGATGGT 170
CysGlnAsnIleThrGluGluPheTyrGlnSerThrCysSerAlaValSerArgGlyTyrLeuSerAlaLeuArgThrGlyTrpT 53
 ATACAAGTGTAGTAACAATAGAGTTGAGCAAAATACAAAAGTAAGTACTGATTCAAAAGTGAATTAATAAAGCA 255
 yrThrSerValValThrIleGluLeuSerLysIleGlnLysAsnValCysLysSerThrAspSerLysValLysLeuIleLysGl 81
 AGAACTGGAAGATACAACAATGCAGTAATAGAATTGCAGTCACCTTATGCAAAATGAACCGGCTTCCTTCAGTAGAGCAAAAGA 340
 nGluLeuGluArgTyrAsnAsnAlaValIleGluLeuGlnSerLeuMetGlnAsnGluProAlaSerPheSerArgAlaLysArg 109
 GGGATACCAGAGTTGATACATTATACAAGAACTCTACAAGAGATTTTATGGGTTAATGGGCAAGAGAGAGATTTT 425
 GlyIleProGluLeuIleHisTyrThrArgAsnSerThrLysArgPheTyrGlyLeuMetGlyLysLysArgLysArgArgPheL 138
 TAGGATTCTTGCTAGGTATTGGATCTGCTATTGCAAGTGGGTAGCAGTGTCCAAAGTACTACACCTGGAGGGAGAGGTGAATAA 510
euGlyPheLeuLeuGlyIleGlySerAlaIleAlaSerGlyValAlaValSerLysValLeuHisLeuGluGlyGluValAsnLy 166
 AATTAAAAATGCACCTGCTATCCACAAATAAAGCAGTAGTTAGTCTATCCAAATGGAGTTAGTGTCTTACTAGCAAAGTACTTGAT 595
 sileLysAsnAlaLeuLeuSerThrAsnLysAlaValValSerLeuSerAsnGlyValSerValLeuThrSerLysValLeuAsp 194
 CTAAGAAGAACTATAGACAAAGAGCTTCTACCTAAAGTTAAACAATCATGATTGTAGGATATCCAACATAGAAACTGTGATAGAAT 680
 LeuLysAsnTyrIleAspLysGluLeuLeuProLysValAsnAsnHisAspCysArgIleSerAsnIleGluThrValIleGluP 223

12/26

FIG. 9A

SUBSTITUTE SHEET

TCCAAACAAAAACAATAGATTGTTAGAAATTGCTAGGGAATTTAGTGTAATGCTGGTATTACCACACCCCTCAGTACATACAT 765
 heGlnGlnLysAsnAsnArgLeuLeuGluIleAlaArgGluPheSerValAsnAlaGlyIleThrThrProLeuSerThrTyrMe 251
 GTTGACCAATAGTGAATTACTATCATAATTAATGATATGCCTATAACGAATGACCAAAAAAGCTAATGTCAAGTAATGTTCAA 850
 tLeuThrAsnSerGluLeuLeuSerLeuIleAsnAspMetProIleThrAsnAspGlnLysLysLeuMetSerSerAsnValGln 279
 ATAGTCAGACACAGAGTATTCCATTATGTCAGTGGTCAAGAAGAGGTCATAGCTTATGTTGTACAATTGCCTATTATGGAG 935
 IleValArgGlnGlnSerTyrSerIleMetSerValVallLysGluGluValIleAlaTyrValIleGlnLeuProIleTyrGlyV 308
 TTATAGACACCCCTGTTGGAACTACACACCTCTCCATTATGCACCACTGATAATAAAGAGGTCAAACATCTGCTTAACTAG 1020
 alIleAspThrProCysTrpLysLeuHisThrSerProLeuCysThrThrAspAsnLysGluGlySerAsnIleCysLeuThrAr 336
 GACAGATCGTGGTGGTATTGTGACAAATGCAGGCTCTGTGCTCTTTTCCACAGGCAGAGACATGTAAAGGTACAATCAAATAGA 1105
 gThrAspArgGlyTrpTyrCysAspAsnAlaGlySerValSerPheProGlnAlaGluThrCysLysValGlnSerAsnArg 364
 GTGTTCTGTGACACAAATGAACAGTTTAACTCTGCCTACTGATGTAACTTATGCAACACTGACATATTCAATACAAAGTATGACT 1190
 valPheCysAspThrMetAsnSerLeuThrLeuProThrAspValAsnLeuCysAsnThrAspIlePheAsnThrLysTyrAspC 393
 GTAAATAATGACATCTAAACTGACATAAGTAGCTCTGTGATAACTTCAATAGGAGCTATTGTATCATGCTATGGGAAGACAAA 1275
 ysLysIleMetThrSerLysThrAspIleSerSerSerValIleThrSerIleGlyAlaIleValSerCysTyrGlyLysThrLy 421
 ATGTACAGCTTCTAATAAAAAATCGTGAATCATAAAGACTTTTCCAAATGGGTGATTATGTATCAAAACAAGGAGTTGACACT 1360
 sCysThrAlaSerAsnLysAsnArgGlyIleIleLysThrPheSerAsnGlyCysAspTyrValSerAsnLysGlyValAspThr 449

13/26

FIG. 9B

14/26

.
 GTATCTGTTGGTAACACACTATATATGTAATAAGCTAGAGGGGAAAGCACTCTATATAAAGGGTGAACCAATTATTAATTACT 1445
 ValSerValGlyAsnThrLeuTyrTyrValAsnLysLeuGluGlyLysAlaLeuTyrIleLysGlyGluProIleIleAsnTyrT 478

 ATGATCCACTAGTGTTCCTTCTGATGAGTTTGATGCATCAATTGCCCAAGTAATGCAAAAATAAACCAAGCCTGGCTTTCAT 1530
 yrAspProLeuValPheProSerAspGluPheAspAlaSerIleAlaGlnValAsnAlaLysIleAsnGlnSerLeuAlaPheI 506

 ACGTCGATCTGATGAGTTACTTCACAGTGTAGATGTAGGAAATCCACCAAAATGTAGTAATTACTACTATTATTATAGTGATA 1615
 eArgArgSerAspGluLeuLeuHisSerValAspValGlyLysSerThrThrAsnValValIleThrIleIleIleValIle 534

 GTTGTAGTGATATTAATGTTAATAGCTGTAGGATTACTGTTTACTGTAAGACCAAGAGTACTCCTTATCATGCTAGGAAAGGATC 1700
 ValValValIleLeuMetLeuIleAlaValGlyLeuLeuPheTyrCysLysThrArgSerThrProIleMetLeuGlyLysAspG 563

 AGCTTAGTGGTATCAACAATCTTTCCTTTAGTAAATGAAATGCATAATGTTTACAATCTAAACCTCAGAATCATAAATGTGATGA 1785
 InLeuSerGlyIleAsnAsnLeuSerPheSerLysEnd 574

 GCTAAATTTTATTAATACATTCAAAAGTTCTATCCGCCCAAGACCTGCATTTTTTATCAGGTCCTTATATAAGCTAACCTTACATGC 1870

 TACACTCAGCTCCATGTTGATAGTTATAT 1899

FIG. 9C

15/26

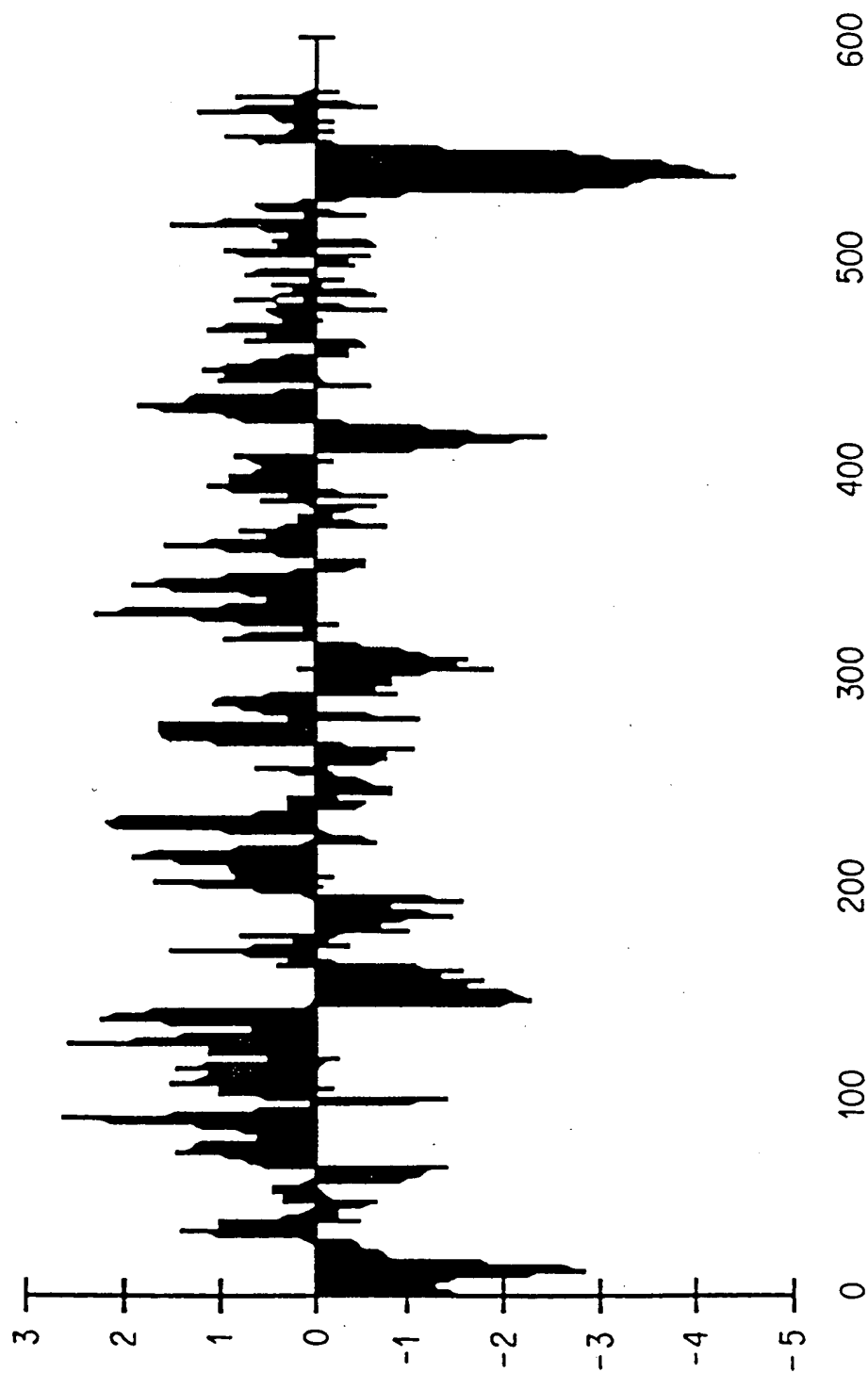


FIG. 10

A2 ELLILKANAITTILTAVTECFASG
 Long ELPILKANAITTILAAVTECFASS
 RSS-2 ELPILKTNAITAILTAVTLCFASG
 18537 ELLIH SSAIFLTLAVNALYL SS
 Bovine MAATAMRMIISIIFISTYMTHTLCQNI

I I I I
 N KE K NG
 N KE K NG
 N KE K NG
 N KETK NG

K K K F

EEFYQSTCSAVSRGYLSALRTGWYTSVVVTIELSKIQKNVCKSTD75

DK K T L ST PTNN R EL RFMN L NA KTNVTLS
 DK K T L ST ANN R EL RFMN I N KTNVTLS
 DK KS T L ST TNN R EL RFMN I N NTNVTLS
 DK K T L T ANN R EA QYMN I T NINDSIS

V V V V
 I I I I

76 VKLIKQELERYNNAVIELQSLMQNEPASFSRAKRGIPELIHYTHNSTKRFYGLMGKKRRRFLGELGIGSAVAS150

V S
 T V S
 I V S
 I V G

Q I KQS S
 Q I KQS
 Q I KQS S
 NNR I QQS T

151 GVALSKVLHLEGEVKNKKNALLSTNKAVVSLNSGVSLTSKVLDLKNYIDKELLPKVNNHDCRISNIETVIEFQQ225

FIG. 11A

18/26

451 SVGNTLYYVKNLEGGKALYIKGEPIINYDPLVFPSPDEFDASIAQVNAKINQSLAFIRRSDELLHSVDVGKSTTNV 525

Q	S	V	F	S	E	K	N	NA	I
Q	S	V	F	S	E	K	H	NA	I
Q	S	V	F	S	E	K	N	NA	I
	N	V		S	E		N	NT	I

526 VITTTIIIVVILMLIAVGLLFYCKTRSTPIMLGKQQLSGINNLSFSK 574

M	I	IL	S	L	A	VT	S	IA	N
M	I	IL	S	L	A	VT	S	IA	N
M	I	IL	S	L	A	VT	S	IA	N
M	I	L	S	I	L	AKN	VT	IA	

FIG. 11C

19/26

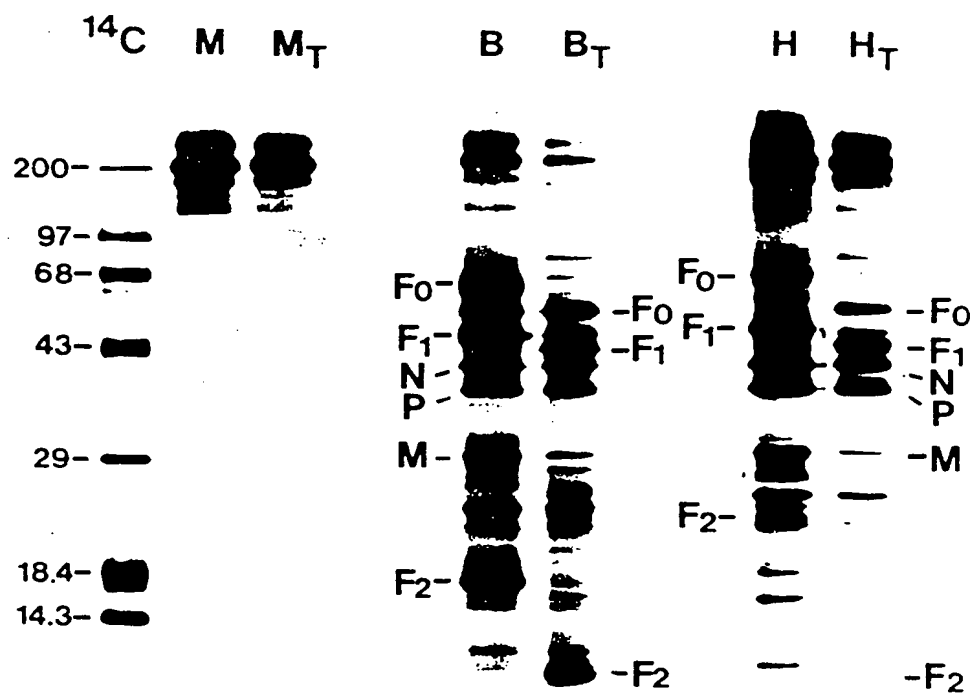


FIG. 12

20/26

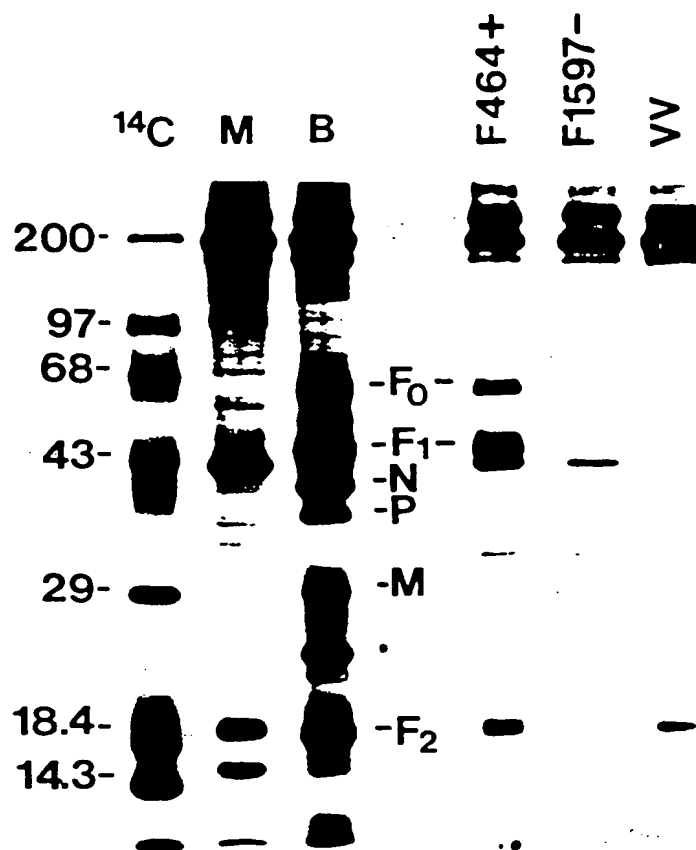


FIG. 13

21/26

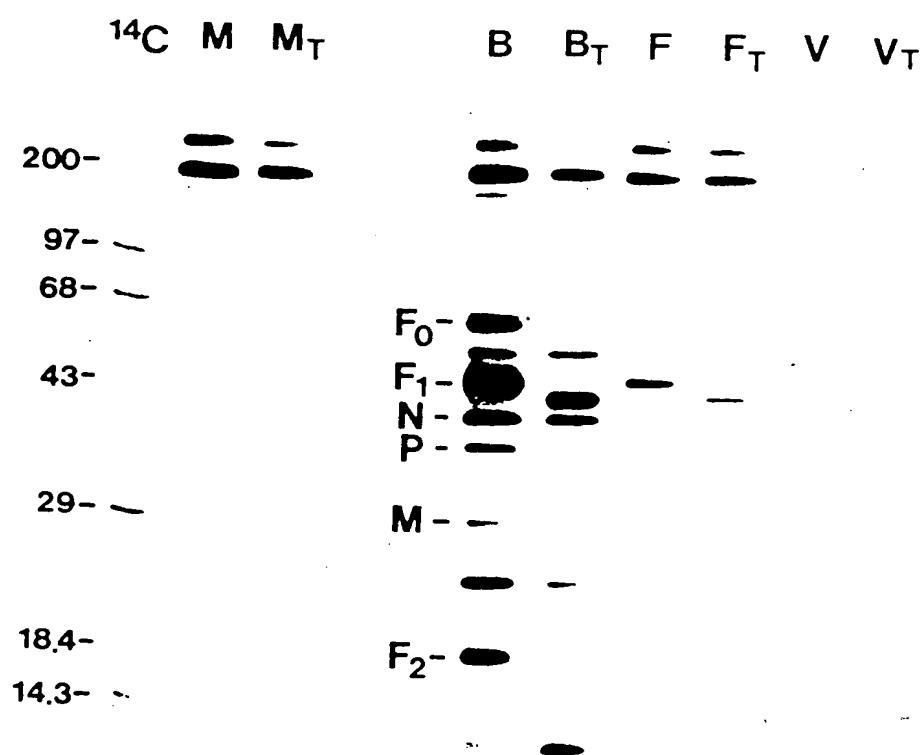


FIG. 14

22/26

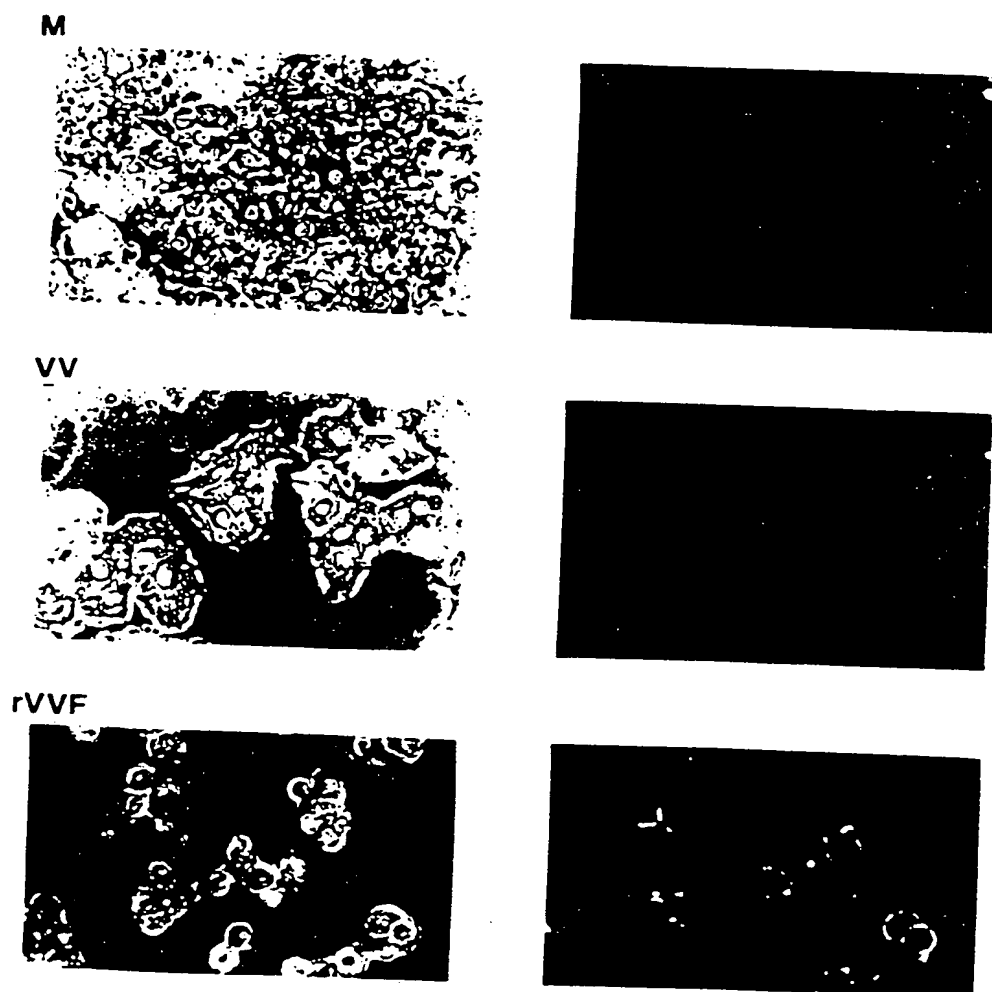


FIG 15

23/26

A			B			C		
Anti Hu G			Anti Bov G			Total		
M	Bov	Hu	M	Bov	Hu	M	Bov	Hu

G

FIG. 16

24/26

1 CAAAATGGC TCTTAGCAAG GTCAAACTAA ATGACACTTT CAACAAGGAC
51 CAACTGTTGT CAACCAGCAA ATATACTATT CAACGTAGTA CAGGTGACAA
101 CATTGATATA CCCAATTACG ATGTGC AAAA ACATCTCAAT AAGTTGTGTG
151 GTATGCTATT AATAACAGAA GATGCCAATC ATAAATTTAC AGGACTGATA
201 GGTATGTTAT ATGCTATGTC CCGATTGGG AGAGAAAGATA CCCTTAAAT
251 ACTCAAAGAT GCAGGCTACC AAGTGAGGGC CAATGGGGTT GATGTGATAA
301 CACATCGACA GGATGTGAAT GGAAAGAAA TGAATTTGA AGTGCTAACA
351 TTAGTCAGCT TAACATCAGA AGTICAAGGT AATATAGAAA TAGAGTCAAG
401 GAAGTCTTAC AAAAAGATGC TAAAGAGAT GGGAGAGGTA GCTCCAGAAT
451 ACAGACATGA CTCTCCTGAT TGTGGTATGA TAGTGCTATG TGTGCTGCT
501 TTGGTTATAA CAAATTAGC AGCAGGTGAT AGGTCAGGCC TCACTGCAGT
551 CATTAGGAGA GCCAACAATG TACTAAGGAA TGAATGAAA CGATACAAAG
601 GACTCATCCC GAAAGATATA GCCAACAGCT TCTATGAAGT ATTTGAAAAG

FIG. 17A

25/26

651 TACCCTCATT ACATAGATGT ATTCGTACAT TTTGGCATTG CTCATCCTC
701 AACTAGAGGA GGTAGTAGGG TAGAAGGAAT CTTTGCAGGG TTATTCATGA
751 ATGCATATGG AGCAGGTCAA GTGATGTAA GATGGGGTGT GCTAGCCCAA
801 TCAGTCAAGA ACATTATGCT TGGTCATGCC AGCGTACAAG CAGAAATGGA
851 ACAGGTTGTA GAAGTCTATG AATATGCACA AAAGTTAGGT GGAGAAAGCTG
901 GTTTTATCA CATACTGAAC AATCCTAAAG CATCATTTGT ATCCITTGACA
951 CAATTCCCCA ACTTCTCTAG TGTAGTCCTA GGCAATGCTG CAGGACTAGG
1001 TATAATGGGT GAGTATAGAG GTACACCAAG AAACCAAGAC TTGTATGATG
1051 CTGCCAAAGC ATATGCAGAA CAACTAAAG AGAATGGGT CATCAATTAC

1101 AGTGTGTTGG ATCTGACTAC AGAGGAATTA GAGGCAATCA AGAACCAATT
1151 GAATCCCCAA GATAATGATG TGGAAATTGT AGTTAATAAA AAAA

FIG. 17B

26/26

1 KMA LSKVKLN DTFNKDQLLS TSKYTIQRST GDNIDIPNYD VQKHLNKLCCG
51 MLLITEDANH KFTGLIGMLY AMSRLGREDT LKILKDAGYQ VRANGVDVIT
101 HRQDVNGKEM KFEVLTLSL TSEVQGNIEI ESRKSYKKML KEMGEVAPEY
151 RHDFPDCGMI VLCVAALVIT KLAAGDRSGL TAVIRRANNV LRNEMKRYKG
201 LIPKDIANSF YEVEFEKYPHY IDVFVHFGIA QSSTRGGSRV EGIFAGLFMN
251 AYGAGQVMLR WGVLA KSVKN IMLGHASVQA EMEQVVEVYE YAQKLGGEAG
301 FYHILNPKA SLLSLTQFPN FSSVVLGNAA GLGIMGEYRG TPRNQDLYDA
351 AKAYAEQLKE NGVINYSVLD LTTEELEAIK NQLNPKDNDV EL*VNKK

FIG. 18

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/05194

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC(5): A61K 39/00; C07H 15/12; C12N 1/00, 1/16, 5/00, 7/00; C07K 3/00, 13/00; C12P 21/06; C12Q 1/70 U.S. CL.: 424/88; 435/5, 243, 235.1, 240.1, 255; 530/350, 387; 536/27		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
U.S. CL.	424/88; 435/5, 243, 235.1, 240.1, 255; 530/350, 387; 536/27	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
AUTOMATED PATENT SEARCH (APS): DIALOG		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	US, A, 4,855,224 (BERMAN ET AL.) 08 AUGUST 1989, see entire document.	12-36, 44-84, 95-111, 116-126, 129
<div style="display: flex; justify-content: space-between;"> <div style="width: 48%;"> <p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 48%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"d" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
28 OCTOBER 1991		30 DEC 1991
International Searching Authority		Signature of Authorized Officer
ISA/US		BRADLEY L. SISSON

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	JOURNAL OF VIROLOGY, Volume 49, NO.2, Issued 1984, COLLINS <u>et al.</u> , "Identification of a tenth mRNA of Respiratory Syncytial Virus and Assignment of Polypeptides to the 10 Viral Genes", pages 572-578 see abstract no. 5554515.	1-11,15-26,37-43, 51-62,73,74,112-115,122
A	JOURNAL OF VIROLOGY, Volume 53, No.4, Issued 1985, Collins <u>et al.</u> , "The Envelope-Associated 22K Protein of Human Respiratory Syncytial Virus: Nucleotide Sequence of the mRNA and a Related Polytranscript", pages 65-71, see abstract no. 5841336.	1-11,15-26,37-43, 51-62,73,74,112-115,122
X	JOURNAL OF VIROLOGY, Volume 62, No.12, Issued 1988, Nicholas <u>et al.</u> , "Mapping an Antibody-Binding Site and a T-Cell-Stimulating Site on the 1A Protein of Respiratory Syncytial Virus", pages 4465-4473, see abstract No. 6977246.	1-11,15-26,37-43, 51-62,73,74,112-115,122
X,P	JAPANESE JOURNAL OF VETERINARY SCIENCE, Volume 52, No.4, Kubota <u>et al.</u> , "Establishment of an Attenuated strain of Bovine Respiratory Syncytial Virus for live Cattle Vaccine", pages 695-703, see abstract no. 1536426.	27-36,63-72,75,76,120,121,123

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

X	US,A,4,790,967 (COMPANS ET AL.) 13 DECEMBER 1988, SEE ENTIRE DOCUMENT.	27-34,63-69,70-72, 75,96-111,120,121, 123
X	US,A, 4,563,419 (Ranki et al) 07 January 1986, see entire document.	85-95,127,128
A,P	US,A, 4,994,368 (Goodman et al) 19 February 1991, see entire document.	85-95, 127,128

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____, because they relate to subject matter ¹² not required to be searched by this Authority, namely:

2. ☐ Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out ¹³, specifically:

3. ☐ Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ²

This International Searching Authority found multiple inventions in this international application as follows:

See attached sheets.

1. ☒ As all required additional search fees were timely paid by the applicant, this International search report covers all searchable claims of the international application. telephone practice

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

BEST AVAILABLE COPY

PCT US91/05194

The inventions are distinct, each from the other because of the following reasons:

Group I, claims 1-11, 37-43, and 112-115, drawn to DNA, classified in Class 536 and Subclass 27.

Group II, claims 12-21, 48-57, and 119, drawn to transformed eukaryotic cell and method of producing recombinant protein, classified in Class 435 and Subclasses 240.1 and 69.1.

Group III, claims 22-26, 58-62, 73, 74, and 122, drawn to viral protein, classified in Class 530 and Subclass 350.

Group IV, claims 27-32, 35, 36, 63-68, 71, 72, 75, 76, 121, and 123, drawn to vaccine, classified in Class 424 and Subclass 88.

Group V, claims 33, 34, 69, 70, and 120, drawn to virus, classified in Class 435 and Subclass 235.1.

Group VI, claims 44-46, 47, 116 and 117, drawn to recombinant bacterium, classified in Class 435 and Subclass 243.

Group VII, claims 77-84 and 124-126, drawn to antibody, classified in Class 530 and Subclass 387.

Group VIII, claims 85-95, 127, and 128, drawn to method of diagnosing, classified in Class 435 and Subclass 5.

Group IX, claims 96-111 and 129, drawn to method of inducing an immune response, classified in Class 424 and Subclass 88.

Group X, claim 118, drawn to recombinant yeast, classified in Class 435 and Subclass 255.

The inventions of Groups I and II are distinct from each other as the invention of Group I may be used as a molecular weight standard as used in

BEST AVAILABLE COPY

Southern blots while the invention of Group II is directed to a life form having utility as a possible source for a protein.

The invention of Group III is distinct from all other Groups as it is directed to a specific chemical composition which has utility as a molecular weight standard as used in Western blots.

The invention of Group IV is distinct from all other Groups as it is the only group drawn to a vaccine, thereby having a unique and defined utility and requiring a different search.

The invention of Groups II, V, VI, and X are distinct from each other as well as from all other groups as said groups are all drawn to different life forms, each having distinct limitations and utilities and requiring separate searches.

The invention of Group VII is distinct from all other groups as it is directed to a distinct chemical composition, requiring a different search from other groups.

The invention of Group VIII and IX are distinct from each other as well as from all other Groups as Groups VIII and IX are directed to distinct methods which utilize different reagents and result in different end products.

Because these inventions are distinct for the reasons given above and: (1) have acquired a separate status in the art as shown by their different classification; (2) because of their recognized subject matter; and (3) the search required for Group IV is not required for Group IX, restriction for examination purposes as indicated is proper.